Vol. 31, No. 4 December 1954

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The Journal of Experimental Biology

EDITED BY
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Published for The Company of Biologists Limited
CAMBRIDGE UNIVERSITY PRESS
CAMBRIDGE, AND BENTLEY HOUSE, LONDON

AGENT FOR U.S.A.
ACADEMIC PRESS INC.,
125, EAST 23RD STREET, NEW YORK, U.S.A.

Price 30s. net *Subscription per volume 84s. net*

THE JOURNAL OF PHYSIOLOGY

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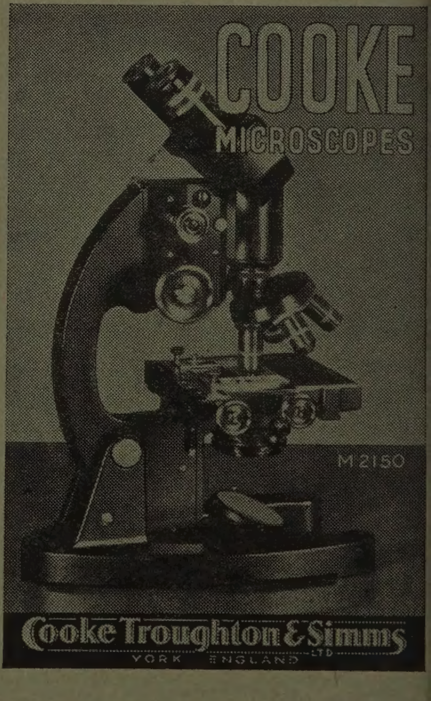

VOLUME 31, NO. 4 DECEMBER 1954

THE COLOUR CHANGE OF THE MINNOW (*PHOXINUS LAEVIS* AG.)

II. EFFECTS OF SPINAL SECTION BETWEEN VERTEBRAE 1 AND 15 AND OF ANTERIOR AUTONOMIC SECTION ON THE RESPONSES OF THE MELANOPHORES

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(*Received* 2 *November* 1953)

I. INTRODUCTION

(*a*) *Previous work*

According to the early work of von Frisch (1911) on the minnow, the nerve fibres responsible for active melanophore aggregation pass from the brain to the spinal cord and leave the latter at about the level of the 15th vertebra to enter the autonomic chain. There they run forwards and backwards, passing out with the spinal nerves and, in the head, with a branch of the trigeminal nerve to supply the melanophores on the surface of the body (Fig. 1*a*). Von Frisch's results indicated that the tonic action of these fibres is controlled by a centre in the medulla which has working against it an inhibitory brain centre whose activity is responsible for melanophore dispersion; he also found evidence for the presence of a subordinate paling centre lying in the more anterior region of the spinal cord.

More recently the opinion has gained ground that in teleosts there may be not only fibres responsible for melanophore aggregation but also fibres controlling active melanophore dispersion. Giersberg (1931) and von Gelei (1942) have claimed to demonstrate the presence of such fibres in the minnow by means of experiments involving treatment with ergotamine and acetylcholine followed by electrical stimulation. The resulting darkening of the animal has been attributed by these workers to excitation of dispersing fibres. According to von Gelei these fibres do not follow the same path as von Frisch's aggregating fibres, but pass from the spinal cord to the autonomic chain with the 1st or 2nd spinal nerve. They then run backwards in the autonomic chain and pass from it to the melanophores (Fig. 1*b*). According to von Gelei's summary of his paper the passage of these fibres from the autonomic chain to the melanophores is effected by way of the spinal nerves, although he does not in fact indicate this in his paper proper or in his diagram. Von Gelei confirmed von Frisch's earlier observation that anterior section of the autonomic chain does not appear to result in abnormal colour changes in the posterior region of the minnow. He therefore concluded that these dispersing fibres play no significant part in the intact animal. His methods of autonomic section are not stated nor are his criteria for normal colour change. The latter would appear to have been based upon the general appearance of the animal when viewed with the naked eye.

In previous papers (Healey, 1940, 1951) the author has shown that after section of the spinal cord of the minnow at some point between the 5th and the 12th vertebrae there is an immediate darkening of the fish regardless of its background. After some time there are once more colour changes in response to illuminated black and white backgrounds and to darkness, but these are very slow compared with those of the unoperated animal. They appear to be controlled by a pituitary hormone causing melanophore aggregation and probably a second pituitary hormone causing melanophore dispersion.

(*b*) *Present work*

Although spinal section in the minnow anterior to the 15th vertebra must result in a disconnexion from the brain of the more peripheral part of the chromatic nervous system which is responsible for melanophore aggregation (Fig. 1*a*), the path of the dispersing fibres indicated by von Gelei (Fig. 1*b*) is not interrupted unless the operation is carried out anterior to the 1st spinal nerve. Since in the author's earlier experiments all spinal sections were behind the level of the 4th vertebra, the possibility arises that von Gelei's fibres play a part in the colour changes of fish operated in this way (von Frisch, private communication).

Before such a possibility can be investigated a further point requires consideration. It has been shown from macroscopic observations (Healey, 1940) that the minnow can change colour slowly not only after spinal section between the 5th and the 12th vertebrae but also when, in addition to this operation, any possible action upon the melanophores of the paling centre in the spinal cord has been eliminated. This spinal paling centre is therefore not essential for colour changes of the operated fish. Nevertheless, the limits of the colour changes of spinal minnows vary so greatly (Healey, 1951) that the possibility cannot immediately be ruled out without further experiment that the level at which the section takes place, regardless of its relation to the point of exit of von Gelei's dispersing fibres, may affect the degree of colour change in that it determines the extent to which the spinal paling centre is still in communication with the melanophores. If von Gelei's fibres are as little effective as he suggests, their influence might well be masked by a slight action of the paling centre.

Thus three questions may be asked:

(i) Can the level of spinal section, posterior to the point of exit of von Gelei's dispersing fibres and anterior to vertebra 15, be correlated with any special chromatic effects which might indicate an activity of the spinal paling centre and obscure any possible action of von Gelei's fibres?

(ii) Does elimination of the spinal paling centre indicate that it is responsible for any effects which might obscure the action of von Gelei's dispersing fibres?

(iii) Do von Gelei's dispersing fibres play any active part in the colour changes of spinal minnows?

An an attempt to answer these questions the chromatic reactions of minnows were observed after section of the nervous system at different levels. The observations were based upon the melanophore index as described in a previous paper (Healey, 1951). The melanophores chosen for observation were the following: the small dermal melanophores of the dorsal region towards the root of the tail (*B*); the large epidermal and dermal melanophores of the lateral stripe and associated dark pattern (L.S.); the small epidermal and dermal melanophores of the dorsal surface, i.e. those lying between the larger melanophores and not contributing to the macroscopic dark pattern (D.S.). These regions probably include the most important types of melanophore groups present on the surface of the minnow and agree fairly well with descriptions of other authors such as that given by Abolin (1925) for minnows from Vienna and Lunz. There are other small scattered melanophores which do not appear to undergo much change in the operated animals but they are so infrequent that they may be disregarded. It is necessary to consider these different regions since von Gelei based his conclusions upon the colour of the fish as a whole and not upon the degree of dispersion of individual melanophores. The darkening which he observed was evidently not great and might have resulted either from a partial dispersion of all the melanophores or from a dispersion of only some melanophore groups. Gross observation of the entire animal, while indispensable for some types of investigation, could not well decide this point. Earlier experiments (Healey, 1951) indicate that these various melanophore regions appear to show approximately the same rate of colour change in the minnow with spinal section between vertebrae 5 and 12, but the degree of dispersion varies among the melanophores of different groups. A non-uniform reaction of the melanophores in the minnow has already been reported by Abolin (1925).

(*c*) *Source and general treatment of fish*

The minnows were obtained from Fron Gogh Pool near Trisant in Cardiganshire. They were all between 5 and 6 cm. long. Their maintenance in the laboratory has already been described (Healey, 1951). The recent heavy chlorination of Aberystwyth tap water has made it poisonous to fish even after very prolonged aeration. Sodium thiosulphate added to the water removes the free chlorine and has no ill effects even when present in considerable excess.

Only fish which appeared to be healthy and which showed a normal macroscopic colour change were used for experiments. These were made in a dark room with black walls illuminated with a 40 W. domestic electric lamp at 1 m. from the fish. The temperature of the water was thermostatically controlled to ±0·2° at 12° C. for the experiments in §II; 14° C. in §§III-VI; 18° C. in §VII. All comparisons of the responses of the melanophores were made using groups of fish at the same temperature and subjected previously to the same treatment apart from differences in operation. Diurnal variations in the degree of melanophore aggregation may produce spurious results. Accordingly, in comparing the effects of different operations the two groups of fish were treated as far as possible together. When this could not be done the two experiments were arranged so that they bore the same relation to the 24 hr. cycle.

The general methods of anaesthetization, operative attack and post-operative treatment have been described (Healey, 1940, 1951). Spurious results were obtained unless the cut on the surface of the animal was restricted as far as possible and kept free from infection. Invasion by the fungus *Saprolegnia* readily occurs. Three minutes in a bath of 3% domestic cooking salt after observations and not less than 4 hr. before an observation sufficed to keep the wounds clean without affecting the chromatic responses.

When the observations on a fish were completed the animal was killed in urethane and then suspended for 2 weeks in Bouin's fluid to harden and decalcify it. After a day in 70% alcohol the site of the operation was determined by dividing the animal by a median vertical longitudinal cut and examining it under a low-power microscope. In doubtful cases serial sections were made. Urethane is better than boiling water for killing the fish, since in it they do not die in twisted positions and can easily be divided with a razor. Bouin's fluid is more suitable than the simple formol-acetic mixture previously used, for the yellow colour which it gives to the tissues makes them more readily distinguishable.

II. COLOUR CHANGES OF MINNOWS AFTER SPINAL SECTION AT VARIOUS LEVELS BETWEEN VERTEBRAE 4 AND 15

The experiments described in this section were carried out in an attempt to find out whether the level of the cut in the spinal cord between vertebrae 4 and 15 can be correlated with any special reactions of the melanophores. Spinal section within these levels interrupts the path of the aggregating fibres (Fig. 1*a*) while leaving the spinal paling centre intact to a varying extent. The path of von Gelei's dispersing fibres (Fig. 1*b*) is not affected.

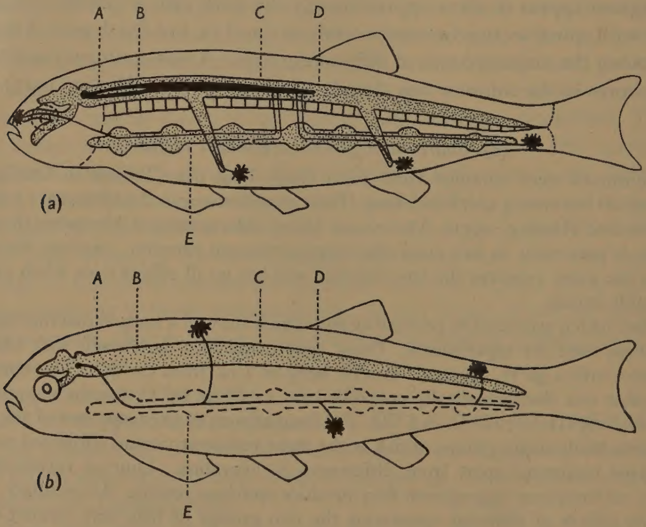

Fig. 1. Paths of chromatic nerve fibres in the minnow. (*a*) aggregating fibres after von Frisch (1911); (*b*) dispersing fibres after von Gelei (1942). *A*, *B*, *C*, *D*, *E* are the regions where the chromatic fibres were cut.

(*a*) *Responses of melanophores of region B from the time of operation to equilibrium on an illuminated white background*

The minnows were placed on a white background after the operation and the mean M.I. values of region *B* were recorded and plotted against time for each vertebral level of spinal section. The general shape of such plots is the same as that already described for spinal minnows operated between vertebrae 5 and 12 (Healey, 1951, fig. 6*b*). (Fig. 2 in the present paper also indicates the form of the plots.) The mean M.I. values and the limits for a few selected times are given in Table 1. An examination of this table shows:

(i) The mean M.I. values reached at a given time by the small groups of fish vary considerably but cannot be correlated with the level of spinal section.

(ii) There is marked individual variation among the fish operated at any one level. The nature of these individual differences is not known.

(iii) Among the six fish operated at vertebra 13 one had a very low M.I. value just after the operation. This persisted and when the fish was later transferred to a black background, although it darkened, it still had a lower M.I. than the other fish of its group (§ II (*b*) (iii)). Similarly, among the five fish operated at vertebra 14 there were two with the low M.I. values of 1·8 and 1·4 at 40 min. When transferred to a black background the first of these darkened normally with the rest of the group, but the other remained at a low M.I. value (§ II (*b*) (iii)). These low M.I. values may be the result of extreme individual variation, but it is much more likely that they are associated with the level of spinal section. The fact that the fish operated at vertebra 15 did not show this low M.I. value does not rule out the second possibility, since in this region of the spinal cord, whether or not we are concerned

Table 1. *Responses of melanophores of region B of minnows subjected to spinal section at different levels and then placed upon an illuminated white background*

Vertebral level of spinal section	No. of fish in group	Mean M.I. values and limits (in brackets) at various times after the operation			
		c. 40 min.	72 hr.	120 hr.	168 hr.
4	1	4·6	1·6	1·4	1·2
5	3	4·0 (2·8–4·6)	1·96 (1·6–2·2)	1·4 (1·4 all)	1·3 (1·2–1·6)
6	4	4·7 (4·2–5·0)	2·0 (1·2–2·4)	1·54 (1·2–2·2)	1·5 (1·2–1·8)
7	5	4·4 (3·4–5·0)	1·7 (1·2–2·0)	1·44 (1·2–1·8)	1·35 (1·0–1·8)
8	2	5·0 (5·0 both)	2·87 (2·6–3·2)	1·5 (1·2–1·8)	1·3 (1·2–1·4)
9	7	4·46 (3·0–5·0)	1·87 (1·0–2·8)	1·7 (1·0–2·6)	1·23 (1·2–1·4)
10	3	5·0 (5·0 all)	3·67 (3·0–4·0)	3·32 (2·0–4·0)	2·72 (1·8–3·6)
13	6	3·7 (1·4–4·8)	1·5 (1·2–2·6)	1·57 (1·2–2·8)	1·35 (1·2–1·8)
14	5	3·15 (1·4–4·8)	1·6 (1·2–2·4)	1·43 (1·2–1·6)	1·3 (1·2–1·6)
15	1	4·4	2·0	1·6	1·2

with an activity of the paling centre, we are concerned with the point or points of exit of the aggregating fibres into the autonomic chain. Von Frisch indicated that these fibres pass over at *about* the level of the 15th vertebra but not always exactly at this level. There is therefore justification for the view that spinal section at levels slightly anterior to vertebra 15 may, in some cases, interfere only partially with the aggregating nerve fibres.

(*b*) *Responses of melanophores of region B following transference of white-adapted operated minnows to an illuminated black background*

The plots of the mean M.I. values of region *B* against time during the white-to-black colour change resemble those already described for spinal minnows operated between vertebrae 5 and 12 (Healey, 1951, fig. 8*b*). (Fig. 4 in the present paper also indicates the form of the plots.) The mean M.I. values and the limits for a few selected times are given in Table 2. No special significance should be attached to the fact that there are two series of fish at some vertebral levels in this table. These were merely observed at different times and could not well be combined. Those groups marked with an asterisk have already appeared in Table 1. An examination of this table shows:

(i) The variations in mean M.I. value reached at a given time by the different groups of fish are still more pronounced but cannot be correlated with the level of spinal section.

(ii) The fish operated at any one level again show marked individual variation.

(iii) The fish operated at the 13th and 14th vertebrae and noted for their low M.I. values in §II(*a*) (iii) still maintain low values on a black background. The suggestion has been made that these may be the result of incomplete section of aggregating fibres. The low M.I. values at 80 hr. of groups 7(*a*), 8(*a*) and 9(*a*), where the section of aggregating fibres must be complete, throws some doubt on this suggestion.

(*c*) *Responses of melanophores of region B from the time of operation to equilibrium on an illuminated black background followed by transference to an illuminated white background*

Experiments on the operation-to-black and the black-to-white colour changes gave results similar in every way to those already presented in §II(*a*) and (*b*), namely:

(i) The plots have the same general shape as those given in an earlier paper (Healey, 1951, figs 6*a* and 8*a*). (Figs. 3 and 5 in the present paper also indicate the form of the plots.)

(ii) There is the same picture of individual variation and of varying mean M.I. values which cannot be correlated with vertebral level.

(iii) In the black-to-white series there is again an indication of more rapid initial paling in some fish operated near the 15th vertebra.

Table 2. *Responses of melanophores of region B of minnows subjected to spinal section at different levels, equilibrated upon an illuminated white background and then placed on an illuminated black background*

(Those groups marked with an asterisk also appeared in Table 1.)

Vertebral level of spinal section	No. of fish in group	Mean M.I. values and limits (in brackets) at various times after placing on black background		
		0 hr.	*c.* 40 hr.	*c.* 80 hr.
4*	1	1·2	4·0	4·8
5*	3	1·4 (1·2–1·6)	3·85 (3·2–4·6)	4·33 (3·6–4·8)
6*a*	2	1·2 (1·0–1·4)	3·9 (3·9 both)	4·1 (3·8–4·4)
6*b*	3	1·38 (1·2–1·6)	3·3 (2·4–3·8)	3·92 (3·6–4·2)
7*a*	3	1·15 (1·0–1·2)	2·35 (1·2–3·8)	2·8 (2·4–3·0)
7*b*	3	1·32 (1·2–1·6)	4·15 (3·4–4·8)	4·32 (4·0–4·6)
8*a*	3	1·45 (1·0–2·4)	2·6 (1·2–4·0)	3·33 (1·8–4·4)
8*b**	2	1·4 (1·2–1·6)	3·54 (2·4–4·6)	4·0 (3·4–4·6)
9*a*	3	1·65 (1·2–2·4)	3·04 (2·4–3·8)	3·74 (2·0–4·6)
9*b*	2	1·2 (1·2 both)	4·0 (3·4–4·6)	4·6 (4·6 both)
10*	2 (1 had died)	3·1 (2·6–3·6)	4·9 (4·8–5·0)	4·8 (4·8 both)
13*	6	1·3 (1·2–1·8)	3·3 (2·0–4·6)	3·5 (2·4–4·6)
14*	5	1·3 (1·2–1·6)	3·54 (1·2–4·8)	3·72 (1·4–4·6)
15*	1	1·2	3·0	3·0

(*d*) *Responses of other melanophores*

Similar records made of the other melanophore regions lead to conclusions which agree with those obtained for region *B*.

These results show that:

(i) The times necessary for the melanophores to reach equilibrium on a given background are of the same order at all the vertebral levels investigated.

(ii) There is great variation in the degree of dispersion of the melanophores of different individuals under similar conditions of operation and background. In general, a fish which readily tends to have a high M.I. value when becoming black-adapted shows less tendency to reach a low M.I. value when becoming white-adapted and vice versa.

(iii) There appears to be no correlation between chromatic behaviour and the level of the spinal section, apart from the possibility of incomplete section of chromatic nerve fibres in the region of vertebra 15: i.e. there is no indication that any activity of the spinal paling centre is being affected by spinal section at the different levels.

III. THE SPINAL PALING CENTRE AND THE COLOUR CHANGE OF MINNOWS SUBJECTED TO SPINAL SECTION BETWEEN VERTEBRAE 3 AND 7

The experiments described in this section were made in an attempt to find out whether total elimination of the spinal paling centre affects the colour changes of minnows whose spinal cords have been cut anterior to the 15th vertebra but posterior to the point of exit of von Gelei's fibres into the autonomic chain. In one group of fish the cut was made at some point between the 3rd and the 7th vertebra (Fig. 1, *B*). In another group each was cut similarly but, in addition, a piece of the spinal cord extending at least from the 12th to the 17th vertebra was completely removed in order to sever connexion between the spinal paling centre and the melanophores (Fig. 1, *C–D*). Two such pairs of groups were placed immediately after the operation on a white and a black background respectively and allowed to reach a steady state. The backgrounds were then reversed. The M.I. values for the various melanophore regions were recorded at known times and the means were plotted. In Figs. 2–5 a smoothed curve is drawn through the means of region *B* and the limits of individual variation for this region are indicated by vertical lines. The means for the melanophores in the other regions are shown by symbols placed at the appropriate M.I. level beside the vertical limit lines for region *B*. Similar wide individual variations were found in these other melanophores but their representation in the figures would have resulted in confusion.

(*a*) *Colour changes from the time of operation to equilibrium on an illuminated white background*

The melanophore dispersion which follows the operation persists for a few days and thus introduces a temporary factor influencing the chromatic behaviour. After the first effects of the operation have worn off the further responses of the melanophores to background changes are much more rapid (Healey, 1940, 1951). Fig. 2 shows the first slow paling on a white background after the operation. In the course of this paling all the melanophores do not behave in the same way.

In group (*a*), in which the spinal paling centre remained intact, (i) the mean M.I. value for the lateral stripe dermis (L.S.D.) melanophores is relatively high throughout; (ii) the mean for the lateral stripe epidermis (L.S.E.) melanophores lies somewhat lower; (iii) the means for regions *B*, the dorsal surface epidermis (D.S.E.) and dorsal surface dermis (D.S.D.) lie still lower and fairly close together.

In group (*b*), in which the spinal paling centre had been eliminated, the mean for L.S.D. is again relatively high but that for L.S.E. is about the same as those of the remaining melanophore regions.

This relative mean M.I. position for L.S.E. is not associated with the presence or absence of the spinal paling centre but appears to be a matter of chance as far as these experiments are concerned. In another series of observations the group with intact paling centre had L.S.E. mean values about the same as those of the *B* and D.S. melanophores, while in a third series the group with paling centre eliminated had mean L.S.E. values clearly above those of the *B* and D.S. melanophores (as in Fig. 2*a*).

While the relative position of the L.S.E. melanophore means thus varies somewhat, the L.S.D. means have been found to be relatively high in repeated experiments. (In individual fish these relations do not always obtain and L.S.D. may be relatively low compared with the other melanophore groups. However, this is not common.)

Direct observation of Fig. 2 shows that the times required to reach equilibrium are of the same order in the two groups of fish. However, a clear comparison of the degree of dispersion of the melanophores at any one time cannot be made immediately by inspection.

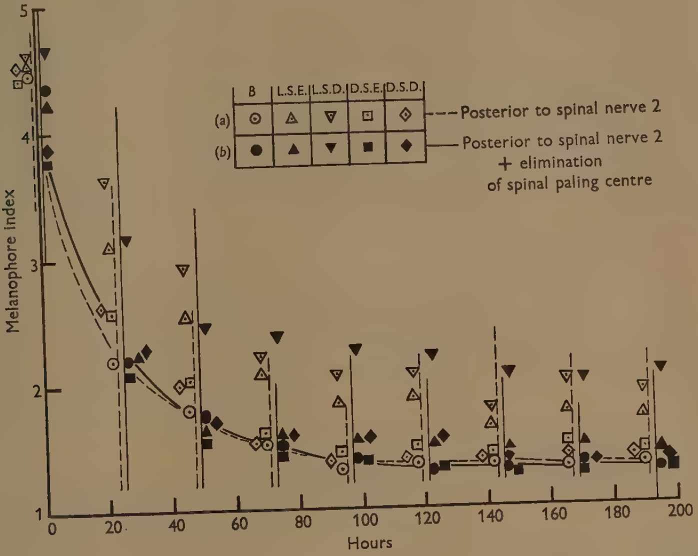

Fig. 2. Responses of the melanophores in two groups of minnows operated as indicated in the figure and placed on an illuminated white background immediately after the operation. All melanophores were read at the same time intervals, i.e. half-way between the pairs of vertical limit lines for region *B*. The first reading was taken about 40 min. after the operation.

If the spinal paling centre were influencing the colour change one might expect group (*a*) to be paler than group (*b*). In fact, in Fig. 2 the reverse applies to nearly all the melanophore regions up to 3 days after the operation and to a number of them after that time. The difference between corresponding groups of melanophores, however, is often not great and the curves for region *B* are nearly coincident. In repetitions of this experiment the *B* regions do not always agree as closely as they do in Fig. 2 and either curve may lie uppermost. Similar relative changes in position are found in the other melanophore regions. Clearly the individual variations found in §II are playing a part. Thus one cannot determine from a mere inspection of the figure to what extent any differences in the chromatic behaviour of the two groups of fish are the result of the different operations.

Fig. 2 is based upon the melanophore index expressed in terms of an arbitrary linear scale and within the limits thus imposed the significance of the differences between the mean M.I. values of the various melanophore regions in the two groups of fish may be tested statistically. For this treatment the *t*-test (Fisher, 1945; Fisher & Yates, 1949, table III) was used. When the probability lay below the 0·05 level the two means were considered to show a significant difference. Such a significant result was then further tested by calculating the variance ratio (Fisher, 1945; Fisher & Yates, 1949, table V). The significance shown by the *t*-test was regarded as confirmed only when the variance ratio gave a non-significant result (less than 5 %).

Tests for significance were applied to the results given in Fig. 2 at 24, 96 and 192 hr. Table 3 (1) shows the levels of probability for the five melanophore regions in the two groups of fish at these times. With one exception they are all well above 0·05, i.e. they indicate no significant differences. The exception is L.S.E. at 24 hr. where the probability is just within the 0·05 level. This is confirmed by the variance ratio which is less than 5 %. This transient significant difference at 24 hr. was not found in two other groups of fish similarly operated. In any case, it is in the opposite direction to that which would be expected were the paling centre exerting any influence.

(*b*) *Colour changes from the time of operation to equilibrium on an illuminated black background*

Since minnows which have undergone simple spinal section show a nearly maximal M.I. when kept on an illuminated black background after the operation, those in which, in addition, the spinal paling centre has been eliminated can not be expected to show a clearly higher M.I. Fig. 3 shows that, if anything, they have a lower M.I. throughout, although Table 3 (2) shows that the difference is not significant. Such a gross operation, involving a severe attack upon so small an animal as a minnow, may cause shock paling which takes some time to disappear.

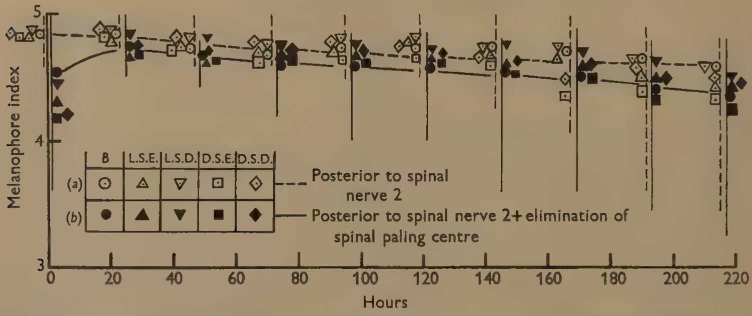

Fig. 3. Responses of the melanophores in two groups of minnows operated as indicated in the figure and placed on an illuminated black background immediately after the operation. All melanophores were read at the same time intervals, i.e. half-way between the pairs of vertical limit lines for region *B*. The first reading was taken about 40 min. after the operation.

Table 3. *Results of statistical treatment expressed as probability (0·05 level of significance)*

Type of operation and colour change involved	Time (hr.)	*B*	L.S.E.	L.S.D.	D.S.E.	D.S.D.
P. to sp. n. 2 and P. to sp. n. 2+P.C. elim.						
(1) Operation→White (8 and 12 fish) (see Fig. 2 and § III (*a*))	24	>0·9	0·05 VR<5%	0·3	0·3	0·5
	96	0·4	0·1	0·4	>0·9	0·9
	192	0·4	0·2	0·5	0·8	0·9
(2) Operation→Black (8 and 8 fish) (see Fig. 3 and § III (*b*))	24	0·2	0·02 VR<5%	0·5	0·02 VR<5%	0·2
	48	0·2	0·1	0·4	0·2	0·1
	216	0·1	0·2	0·2	0·1	0·1
(3) White→Black (8 and 10 fish) (see Fig. 4 and § III (*c*))	5, 6	>0·9	>0·9	0·9	0·8	0·6
	70, 74	0·2	0·5	0·9	0·6	>0·9
(4) Black→White (8 and 8 fish) (see Fig. 5 and § III (*c*))	4	0·5	0·6	0·5	0·9	0·8
	16	0·4	—	—	—	—
	26	—	0·7	0·4	0·3	0·3
	55	0·6	0·7	0·4	0·2	0·5
A. to sp. n. 1 and A. to sp. n. 1+P.C. elim.						
(5) Operation→White (8 and 8 fish) (see § IV)	24	0·8	0·9	0·9	0·6	0·6
	96	0·9	>0·9	0·8	0·6	0·5
	192	0·9	0·8	0·8	0·9	>0·9
(6) White→Black (8 and 8 fish) (see § IV)	6	0·8	0·6	0·6	0·8	>0·9
	72	0·7	0·2	0·05 VR<5%	0·2	0·6
P. to sp. n. 2+P.C. elim. and A. to sp. n. 1+P.C. elim.						
(7) Operation→White (12 and 8 fish) (see § V)	24	>0·9	0·05 VR<5%	0·3	0·2	0·4
	96	0·4	0·02 VR>5%	0·2	0·05 VR>5%	0·1
	192	0·2	0·2	0·5	0·02 VR>5%	0·2
(8) Operation→Black (8 and 8 fish) (see § V)	24	0·1	>0·9	0·8	0·5	0·8
	192	0·7	0·05 VR<5%	0·3	0·8	0·8
(9) White→Black (10 and 8 fish) (see § V)	5, 6	0·7	0·8	0·8	0·8	0·6
	24¼, 27	0·4	0·2	0·3	0·5	0·7
	70, 74	0·3	0·3	0·6	0·2	0·3
(10) Black→White (8 and 8 fish) (see § V)	7¾	0·5	>0·9	0·7	0·3	0·6
	55	0·7	0·9	0·7	0·8	0·8
A. to sp. n. 1 and P. to sp. n. 2+P.C. elim.						
(11) Operation→White (8 and 12 fish) (see § VI)	24	0·8	0·05 VR<5%	0·3	0·3	0·7
	96	0·4	0·01 VR<5%	0·5	0·1	0·3
	192	0·4	0·02 VR<5%	0·4	0·1	0·2
(12) White→Black (8 and 10 fish) (see § VI)	6, ·5	0·9	>0·9	0·9	0·6	0·6
	72, 74	0·4	>0·9	0·4	0·9	0·6
P. to sp. n. 2+A. aut. sectn. and P to sp. n. 2+partial operation						
(13) Operation→White (11 and 12 fish) (see § VII)	24	0·5	>0·9	0·3	0·7	0·9
	72	0·3	0·8	0·7	0·3	0·4
	120	0·1	0·2	0·8	0·02 VR<5%	0·05 VR<5%
	168	0·1	0·2	0·7	0·05 VR<5%	0·05 VR<5%
(14) White→Black (9 and 11 fish) (see § VII)	6½	0·4	0·9	>0·9	0·7	0·5
	48	0·9	0·9	0·6	0·7	0·9
(15) Black→White (9 and 11 fish) (see § VII)	6½	0·2	>0·9	0·6	0·8	0·5
	48	0·3	0·4	0·3	0·3	0·2
	96	0·4	0·4	0·4	0·5	0·4

This might explain the initial rise in M.I. in Fig. 3*b*. Again, the severity of the attack on the spinal cord and neighbouring tissues may be responsible for the gradually decreasing M.I. value which appears later in fish without the paling centre, in that it results in progressive physical deterioration. Table 3 (2) shows a transient significant difference for L.S.E. and D.S.E. at 24 hr. (unconfirmed in other experiments), but otherwise no significant difference up to 9 days. Such non-significant differences persist up to 14 days as long as the fish are very carefully tended. Thereafter the condition of the fish is no longer trustworthy. For this reason all experiments involving elimination of the spinal paling centre were completed within this time. (On a white background this effect cannot be detected after equilibrium has been reached because the M.I. is, in any case, low. However, spurious M.I. values for intermediate stages in black-to-white and white-to-black colour changes may readily be obtained if the precautions of this time limit and of careful maintenance of health are not observed.)

Fig. 3 shows, further, that on a black background all the melanophores of the various regions are dispersed to such an extent that differences between them are not very apparent. Nevertheless, the L.S.D. region still tends to show the highest mean M.I. values.

(*c*) *Colour changes following transference of white-adapted operated minnows to an illuminated black background and the reverse*

Fig. 4 and Table 3 (3) show the white-to-black colour change results. Fig. 5 and Table 3 (4) show those of the black-to-white colour change. The same general relations exist between the mean M.I. values of the different melanophore regions as were seen in Fig. 2. The L.S.D. region is again more dispersed than the others, especially in the lower M.I. ranges. No significant differences occur.

Within the limits of a statistical treatment based upon the melanophore index it may therefore be concluded that the spinal paling centre is playing no part in these colour changes.

IV. THE SPINAL PALING CENTRE AND THE COLOUR CHANGE OF MINNOWS SUBJECTED TO SPINAL SECTION ANTERIOR TO SPINAL NERVE 1

Similar experiments to those described in §III were made with minnows whose spinal cords were cut anterior to the 1st spinal nerve (Fig. 1, *A*). In one group this operation alone was carried out while in another group, in addition, the spinal paling centre was eliminated (Fig. 1, *C–D*). (Removal of spinal cord from vertebrae 12–17 inclusive.)

Table 3 (5) and (6) shows the results of the statistical treatment of the operation-to-white and the white-to-black colour changes. In the former the plots resembled those in Fig. 2 with regard to general M.I. levels at given times and time required to reach equilibrium. No significant differences were found. In the latter the plots resembled those in Fig. 4. Here a difference just within the 0·05 level of significance was found for L.S.D. at 72 hr. However, the group with elimination of the

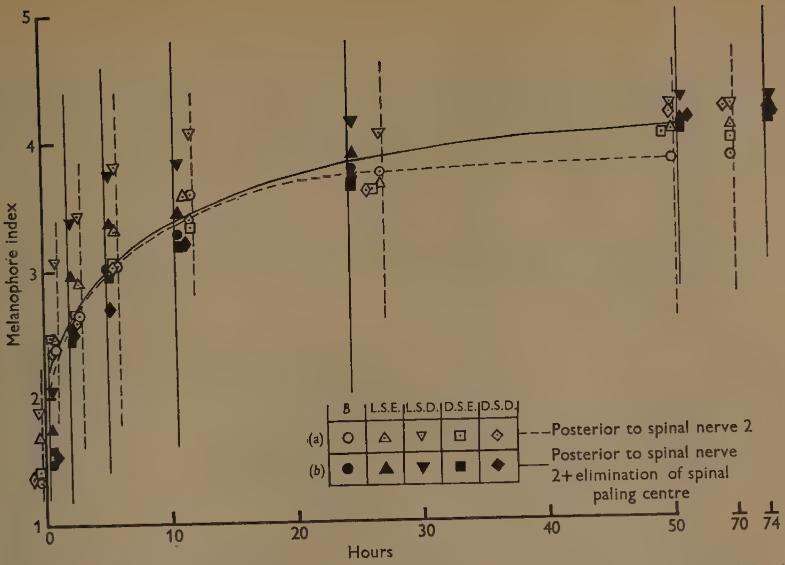

Fig. 4. Responses of the melanophores in two groups of minnows operated as indicated in the figure. After the operation they were left for 9 days on an illuminated white background and then placed upon a black background, this last colour change being shown in the figure. The times of the readings are indicated by the positions of the vertical limit lines excepting for the first reading in each series which is at 0 hr.

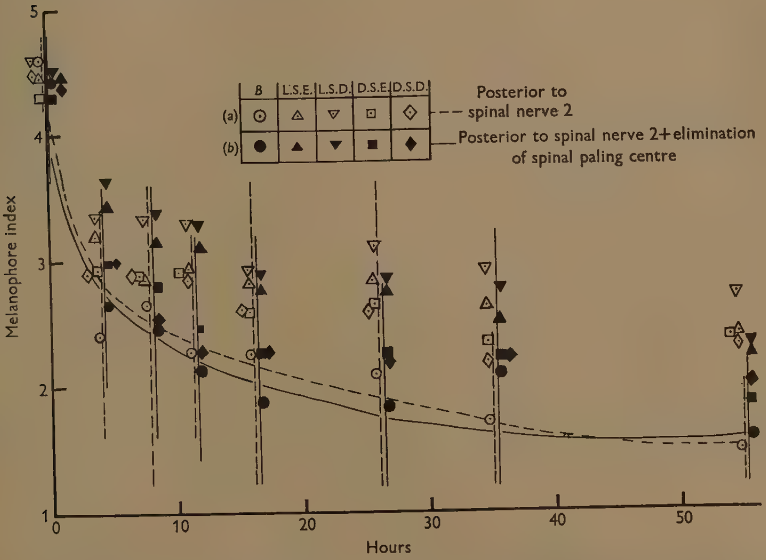

Fig. 5. Responses of the melanophores in two groups of minnows operated as indicated in the figure. After the operation they were left for 9 days on an illuminated black background and then placed upon a white background, this last colour change being shown in the figure. All melanophores were read at the same time intervals, i.e. between the pairs of vertical limit lines.

spinal paling centre had the lower mean M.I. so that there was no indication from this result that this centre was having any influence upon the state of the melanophores. Possibly the adverse effects of the more severe operation (mentioned in §III (*b*)) were beginning to play a part.

The conclusions reached in §III are thus confirmed.

V. COLOUR CHANGES FOLLOWING SPINAL SECTION ANTERIOR TO SPINAL NERVE 1 COMPARED WITH THOSE FOLLOWING SECTION POSTERIOR TO SPINAL NERVE 2, THE SPINAL PALING CENTRE BEING ELIMINATED IN BOTH CASES

The results of §§III and IV indicate that the spinal paling centre does not play a significant role in these colour changes of spinal minnows. Nevertheless, in order to rule out any possibility of action by this centre, it was eliminated (Fig. 1, *C–D* removed) in all the fish used for the following experiments. These were concerned with comparing the colour changes following spinal section anterior to the 1st spinal nerve (Fig. 1, *A*, i.e. von Gelei's fibres interrupted) with those following spinal section posterior to the 3rd spinal nerve (Fig. 1, *B*, i.e. von Gelei's fibres intact). As in §III, four pairs of plots were obtained: operation-to-white, operation-to-black, white-to-black and black-to-white. The relative positions of the mean M.I. plots for the various melanophore regions were the same as those already described in §III. The times required to reach the various equilibrium conditions and the general shapes of the plots closely resembled those of the corresponding plots in Figs. 2–5.

Table 3 (7) indicates that the operation-to-white groups showed significant differences for L.S.E. at 24 and 96 hr. (the latter not confirmed by the variance ratio) and for D.S.E. at 96 and 192 hr. (both unconfirmed by the variance ratio). These differences were, however, in the wrong direction with regard to any possible dispersing effect of von Gelei's fibres. Further, inspection of the black-to-white results (Table 3 (10)) shows no significant differences even at 55 hr. when equilibrium to a white background was almost complete, i.e. at a time when one might expect the chromatic condition of the fish to be similar to that at 192 hr. in the operation-to-white series. The significant results in the operation-to-white series should therefore be regarded with reserve.

Table 3 (8) indicates that the operation-to-black groups showed a transient significant difference for L.S.E. at 192 hr. (confirmed by the variance ratio). There was no significant difference at 168 hr. (probability 0·3) and 216 hr. (0·2).

The white-to-black (Table 3 (9)) and, as mentioned above, the black-to-white groups showed no significant differences.

Within the limits of the methods employed, the results of this section indicate that von Gelei's dispersing fibres are playing no part in these colour changes.

VI. COLOUR CHANGES FOLLOWING SPINAL SECTION ANTERIOR TO SPINAL NERVE 1 COMPARED WITH THOSE FOLLOWING SECTION POSTERIOR TO SPINAL NERVE 2 COMBINED WITH ELIMINATION OF THE SPINAL PALING CENTRE

A further check may be made by testing statistically the differences between similar melanophore regions in two groups of fish already considered separately in §§III–V. In the first group the spinal cord was cut anterior to the 1st spinal nerve (Fig. 1, *A*), i.e. the spinal paling centre was intact and could exert its aggregating influence, if any, unopposed by von Gelei's dispersing fibres. In the second group the spinal cord was cut posterior to the 2nd spinal nerve (Fig. 1, *B*) and the spinal paling centre was eliminated (Fig. 1, *C–D* removed), i.e. von Gelei's fibres could exert their dispersing influence, if any, unopposed by the spinal paling centre.

Table 3 (11) indicates that the operation-to-white colour changes showed no significant differences excepting for L.S.E. These were in the opposite direction to that which would be expected if von Gelei's fibres or the paling centre were active and cannot therefore be taken to indicate such an action. The white-to-black colour changes (Table 3 (12)) showed no significant differences.

The conclusions of §§III, IV and V are thus confirmed.

VII. COLOUR CHANGES FOLLOWING SPINAL SECTION BETWEEN VERTEBRAE 3 AND 6 COMBINED WITH SECTION OF THE AUTONOMIC CHAIN AT ABOUT THE LEVEL OF VERTEBRA 10, THE SPINAL PALING CENTRE REMAINING INTACT

These experiments were made in order to check the previous conclusions. In one group of minnows the operations leading to autonomic section (Healey, 1940) were carried out on each animal up to the point at which the autonomic chain was fully exposed but without actually cutting it. The wound was then sewn up and, after recovery from the anaesthetic, the fish was tested for normal macroscopic colour change. If this was satisfactory its spinal cord was cut between vertebrae 3 and 6 (Fig. 1, *B*). In the other group of fish the complete operation of autonomic section was carried out at about the level of the 10th vertebra (Fig. 1, *E*). As soon as it recovered from the anaesthetic each fish was tested on black and white backgrounds to make sure that the operation had been successful. When it was certain that the anterior part of the body remained permanently dark the spinal cord was cut between vertebrae 3 and 6 (Fig. 1, *B*). Such section of chromatic fibres, both in the autonomic chain and in the spinal cord, is followed, regardless of background, by dispersion of the melanophores distal to the point of section; distal, that is, with regard to the distribution of von Frisch's aggregating fibres (Fig. 1*a*). After some time there is gradual recovery of background responses and slow, hormonally controlled colour changes are possible (Healey, 1940, 1951). In order that such a recovery from the autonomic cut, involving the body region anterior to the point of autonomic section, should not be out of step with recovery from the spinal cut, involving the region posterior to the point of autonomic section, and thus result in a difference in colour between the two regions, the second operation was performed as soon after the first as possible.

Thus, in both groups of fish the spinal cord was cut (Fig. 1, *B*), the spinal paling centre remained intact and the autonomic chain was exposed. They only differed in that the autonomic chain itself was cut in the second group to interrupt von Gelei's fibres (Fig. 1, *E*).

The various melanophore regions were observed as before and the results were plotted. Again the plots agreed closely with Figs. 2–5 in all respects. The results of the statistical treatment are seen in Table 3 (13), (14) and (15). There are no significant differences between corresponding melanophore regions excepting in the operation-to-white colour change for D.S.E. and D.S.D. at 120 and 168 hr. The differences here are in the direction which one would expect if von Gelei's fibres were causing any melanophore dispersion. Since, however, these same melanophore regions showed no significant differences during the later black-to-white colour change when they were once more nearing the condition of equilibrium to a white background, these earlier significant differences may be regarded as transitory.*

In addition to microscopic observations on the melanophores, frequent observations were made on the general appearance of the fish to the naked eye. In minnows operated in this manner the portion of the body anterior to the autonomic cut is under the influence (if any) of von Gelei's dispersing fibres but isolated from any possible influence of the spinal paling centre. The portion of the body behind the autonomic cut is isolated from any possible influence of von Gelei's fibres but under the influence (if any) of the spinal paling centre. If either von Gelei's fibres or the spinal paling centre are affecting the colour changes of these fish one might expect that the region of the body anterior to the autonomic cut would be darker than the region posterior to it. In fact, no such difference in colour was ever detected. This would seem to provide most definite confirmatory evidence that the spinal paling centre and von Gelei's fibres are playing no part in the colour changes of these spinal minnows.

VIII. CONCLUSION

Within the limits of the methods employed it appears that no significant part is played either by the spinal paling centre or by von Gelei's dispersing fibres in the colour changes in response to illuminated backgrounds of spinal minnows operated at any level anterior to vertebra 15. These colour changes must therefore be controlled entirely by hormones. This supports the conclusions of earlier work (Healey, 1940, 1951).

From this it cannot be concluded that von Gelei's fibres play no part in such colour changes in the normal unoperated animal, since the reactions of the melanophores of spinal minnows may be profoundly altered by the operation.

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* Also, the factors involved in the first paling of the freshly operated animal on a white background are not the same as those involved in the later black-to-white colour change.

SUMMARY

1. Minnows were subjected to spinal section at different levels between vertebrae 4 and 15, and the degree of dispersion of all the main melanophore regions was recorded in terms of the melanophore index.

2. Records were made of the times required to reach equilibrium (*a*) when the fish were placed after the operation on a black or white illuminated background, and (*b*) when the fish at equilibrium on a black or white background were subjected to background reversal.

3. These records show that the times necessary for the melanophores to reach equilibrium on a given background are of the same order at all the vertebral levels investigated.

4. There is considerable variation in the degree of dispersion of similar melanophore regions of different individuals under the same conditions of operation and background. The nature of these individual differences is not known.

5. Apart from the possibility of incomplete section of chromatic nerve fibres near the 15th vertebra, there appears to be no correlation between the chromatic behaviour and the level of the operation; i.e. there is no indication that any activity of the spinal paling centre is being affected by spinal section at the different levels.

6. The results of experiments involving the elimination of the spinal paling centre were tested statistically. Within the limits of this treatment, based upon the melanophore index, it was concluded that the spinal paling centre plays no part in these colour changes.

7. Spinal section was carried out anterior to the 1st spinal nerve in order to interrupt the path of von Gelei's dispersing fibres. Statistical treatment of the results indicates that these fibres are playing no part in these colour changes.

8. Experiments involving combined spinal and autonomic section confirm the conclusions given in paragraphs 6 and 7.

9. Since no activity of the nervous system arising from the spinal paling centre or resulting from the background through von Gelei's dispersing fibres appears to be involved, the colour changes of these spinal minnows in response to illuminated backgrounds must be controlled by hormones alone.

10. In these spinal fish the various melanophore regions do not all react equally in terms of the melanophore index. Thus, those of the lateral stripe and associated dark pattern tend to have relatively higher M.I. values under all conditions.

The author wishes to express his thanks to Prof. T. A. Stephenson, F.R.S., for providing facilities which made this work possible; to Mr Percy George of the Department of Economics, University College of Wales, for his kindness in advising on the statistical treatment; to his wife for much help in the treatment and discussion of results.

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THE ABSORPTION OF WATER BY WOODLICE

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(*Received 2 January* 1954)

INTRODUCTION

Woodlice lose water rather rapidly by transpiration into unsaturated air (Edney, 1951). In nature, some loss of water is bound to occur in this way, for they are not always exposed to saturated air, and the present paper reports the results of an investigation into the means available for replenishing such lost water.

It is clear that woodlice absorb water when eating moist food, but partially desiccated animals, left in the presence of potato dice, regain their original weight even when the food is uneaten. It is not clear, in these circumstances, whether water is absorbed from the moist surface of the food (and if so by what route), or from the moist air close to the surface.

Partially desiccated woodlice can also be observed to take up water from droplets or from wet filter-paper by immersion of either the mouthparts or the terminal appendages (uropods). Some of this water at least flows along the capillary channels on the ventral surface which form the *Wasserleitungssystem* first described by Verhoeff (1917, 1920). Whether any of this water enters the alimentary canal has been an open question.

The problems are: (i) can woodlice absorb water vapour from moist air (as some insects do); (ii) do they imbibe liquid water and/or absorb water from damp surfaces, and if so, how?

Four species, chosen to cover as far as possible the range of terrestrial habitats of the group, were used for this investigation. They were *Ligia oceanica* (a littoral species), *Oniscus asellus*, *Porcellio scaber* and *Armadillidium vulgare*. The last three are generally thought of as occurring in progressively drier habitats.

EXPERIMENTS

(*a*) *Absorption from moist air*

Animals were partially desiccated by exposure to dry air at *c.* 18° C. for 1 hr.*, and allowed to recover in stirred air whose humidity was controlled at 98% by the appropriate sulphuric acid and water mixture. Woodlice of all four species continued to lose weight in these conditions.

There is little point in trying to find whether water can be absorbed from humidities higher than this. Apart from the considerable experimental difficulties, woodlice in natural conditions exposed to humidities above 98% are also likely to be exposed to liquid water, as a result of local condensation. For the present, then, it is sufficient to know that they are not capable of increasing their water content by absorbing water vapour even in relative humidities as high as 98 %.

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* In what follows, a 'desiccated' animal means one which has suffered this treatment, not a completely dry specimen.

(*b*) *Absorption of liquid water*

Desiccated woodlice were exposed for 90 min. to filter-paper liberally moistened with a dilute solution of eosin in water, in a Petri dish. They were then dissected to expose the gut, when imbibition, if it had occurred, was revealed by a red colour.

Colour was never seen in the mid-gut, but the wall of the mid-gut is more opaque than that of either the fore- or the hind-gut, so that it was impossible to be sure that no water had passed into or through this region. Consequently, the presence of red colour in the hind-gut could not be taken as evidence of imbibition through the anus. However, when the mouth was blocked, by electro-thermal cautery, no colour at all was ever seen in the fore-gut, so that a red hind-gut in these circumstances was conclusive evidence of anal imbibition. On the same grounds, the presence of red colour in the fore-gut was evidence of oral imbibition. The obvious step of blocking the anus was attempted, but it is a more difficult operation, calling for extensive cautery in order to ensure success, and only a small proportion of animals survived. Anal blockage was therefore discarded as unsatisfactory. The results of these experiments are shown in Table 1.

Table 1. *The site and amount of water uptake by woodlice exposed to free eosin water for* 90 *min. after desiccation in dry air at approximately* 18° C. *for* 1 *hr.*

Species	Condition	No. of animals used	Colour (denoting absorption of water) present in				Gain in weight as % of post-desiccation weight. Mean of 12 records and standard deviation	Remarks
			Fore-gut only	Hind-gut only	Both	Neither		
Ligia oceanica	Intact	17	Nil	12	4	1	3·8 (1·9)	All gained
	Mouth blocked	27	Nil	19	Nil	8	2·3 (1·7)	1 lost, 1 showed no change
Oniscus asellus	Intact	41	21	Nil	18	2	6·4 (3·0)	All gained
	Mouth blocked	40	Nil	32	Nil	8	1·8 (2·3)	3 showed no change
Porcellio scaber	Intact	35	14	Nil	20	1	2·2 (1·0)	All gained
	Mouth blocked	32	Nil	26	Nil	6	4·6 (4·1)	All gained
Armadillidium vulgare	Intact	10	3	Nil	7	Nil	4·3 (2·2)	All gained
	Mouth blocked	7	Nil	6	Nil	1	3·9 (1·6)	All gained

In order to estimate the amount of water absorbed, twelve animals of each species, and with either free or blocked mouthparts, were marked individually and weighed after desiccation and again after 90 min. exposure to eosin water. Before the second weighing they were carefully dried to remove externally adherent water. Drying, which was done with filter-paper, introduced an error in successive test weighings of less than 0·5 % of the original weight in either direction, and it may therefore be neglected.

The results of these experiments are also shown in Table 1. All the animals which were weighed before and after exposure to free water showed an increase in weight, whether the mouth was blocked or not. As regards the site of absorption, animals of each species were capable of imbibing water through the mouth and through the anus, since at least some intact animals showed a red fore- and hind-gut, whereas animals with the mouth blocked never showed colour in the fore-gut though the hind-gut was frequently coloured. Absorption through the anus was much more common than through the mouth in *Ligia*, while the reverse was true of the other three species, for no intact specimen of *Ligia* showed a red fore-gut only, and no intact specimen of the other species showed a red hind-gut only.

A parallel series of experiments was carried out on recently killed animals, but they showed no signs of imbibition into any part of the gut—the process was therefore an active one, not a passive capillary flow.

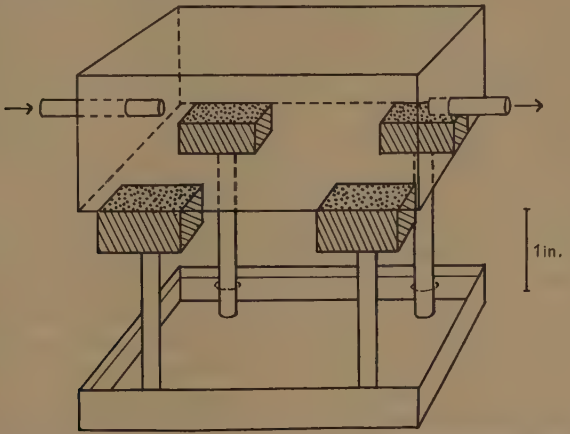

Fig. 1. Perspex container, through which a stream of air may be passed. Four plaster surfaces are let in to the floor and kept damp by capillary suction of water from below.

(*c*) *Absorption from damp surfaces*

Desiccated animals, some with free and some with blocked mouthparts, were weighed and exposed for 90 min. to damp plaster surfaces let in to the floor of a Perspex box through which a slow stream of unsaturated air (*c.* 85% R.H.) was passed. Four plaster surfaces were provided, each approximately 1 in.^{2} in area; the capillary spaces in the plaster were permanently filled from a source of water underneath. The apparatus is shown in Fig. 1. So far as hygrometric factors are concerned, it probably imitates natural conditions reasonably well.

The results are contained in Table 2, and it is clear that intact animals of all the species used, save *Ligia*, gained weight by absorption of water. They were in good condition after such exposure, even when it was prolonged for several hours.

Animals with blocked mouths all lost weight, but it cannot be said on these grounds alone that no water was imbibed through the anus, for such absorption might be small and masked by transpiration. However, no evidence of anal imbibition was found when coloured water was used in this experiment, neither was there any increase in weight if blocked animals were exposed to plaster in saturated air. It can, therefore, be stated with confidence that imbibition in these conditions occurred by mouth only.

Table 2. *Change in weight of woodlice exposed to damp plaster surfaces in slowly moving air at approximately* 18° C. *and* 85 % R.H.

Species	Condition	No. of animals showing			Mean (and standard deviation) change in weight as % of post-desiccation weight
		Loss	No change	Gain	
Ligia oceanica	Intact	9	Nil	1	−1·5 (0·3)
	Mouth blocked	10	Nil	Nil	−6·5 (2·4)
Oniscus asellus	Intact	Nil	Nil	15	+4·5 (2·4)
	Mouth blocked	10	Nil	Nil	−6·3 (4·0)
Porcellio scaber	Intact	Nil	Nil	10	+3·3 (1·6)
	Mouth blocked	12	Nil	Nil	−2·7 (1·0)
Armadillidium vulgare	Intact	Nil	Nil	13	+4·0 (2·0)
	Mouth blocked	8	4	Nil	−1·4* (0·5)

* Those which showed no change are included in this mean.

In *Ligia* there was a loss of weight even in intact animals. This may result partly from the fact that *Ligia* normally absorbs water through the anus, and (as in the other three species) is unable to do so unless a free surface of liquid water is available. But this is unlikely to be the complete answer, for one specimen actually gained weight. It is likely that the loss of water by transpiration from *Ligia* in these conditions is usually greater than the gain of water from a damp surface, whereas in the other species the reverse is true. It is known (Edney, 1951) that *Ligia* loses water by transpiration more rapidly than the other species, and it is suggestive that the other three species stand in the same order as regards loss of weight in the present experiments as they do in respect of the transpiration rates established earlier.

Control experiments were again performed, using dead animals, and these all showed a loss of weight even though left in contact with the damp surface. Imbibition from damp surfaces is, therefore, an active process, as is the absorption of free water.

Intact animals which were exposed in the same box, but with dry plaster surfaces, continued to lose weight and eventually died. It can therefore be said that the presence of a damp surface is sufficient to ensure survival, even though the surrounding air is unsaturated.

(*d*) *Absorption through the integument*

An attempt was made to find whether water can be absorbed through the integument. The method adopted was to cauterize both mouth and anus and then to expose the animals to eosin water. As mentioned above, the effective blocking of the anus necessitates rather extensive cautery, and the small number of animals which survived the double operation were not always in good condition. They were weighed and exposed to eosin water, however, with the following results: Of fifteen *Porcellio scaber*, fourteen showed a mean gain of 2·5 % and one a loss of 1·5 %; of ten *Oniscus asellus*, three showed a mean gain of 2·2 %, five showed no change, and two showed a mean loss of 2 %; of six *Ligia oceanica* three showed a mean gain of 2·3 % and three a mean loss of 1·7 %. There is, therefore, little evidence either way, and the experiment was discontinued until a more satisfactory technique could be devised.

DISCUSSION

There are several statements in the literature to the effect that different species of woodlice inhabit environments of different degrees of dryness (refs. in Edney, 1954). Heeley (1941*b*, 1942) went so far as to state that the distribution of different species could be predicted from a knowledge of the humidity of the surroundings. The same author (1941*a*) stated that in the laboratory the optimum humidity for survival of various species varied from 84 % (*Trichoniscus pusillus*) to 65 % (*Armadillidium vulgare*).

It has been difficult to reconcile these observations with the results of Miller (1938) and Edney (1951). The former measured the period of survival of several species in different saturation deficits and found that 100 % R.H. was optimum at all temperatures for all species. This is not true for really high temperatures near the lethal point, for it has been found (Edney, 1951) that survival is increased, at any rate for short periods, in unsaturated air, because evaporation then lowers the body temperature. It remains true, however, that all species lose water by transpiration in all but saturated air. Miller suggested that woodlice retire by day to cool damp crevices where the air is saturated, and emerge only at night when the air temperature falls and the saturation deficit is therefore low. No doubt this is an important part of the answer.

The results of the present experiments take us a step further, for they show that although water is lost in unsaturated air, the balance may be restored by imbibition, through the mouth at least, from damp surfaces. Evidence is insufficient at present to say how damp the surface must be, or what air humidity is thus rendered tolerable. It seems likely however (and there is some supporting evidence in the present work) that given a damp surface, species which lose less water by transpiration, such as *A. vulgare*, will be able to balance imbibition against transpiration in greater saturation deficits than others. This may go some way towards explaining the various optimum humidities found by Heeley for different species, for his woodlice were living in Petri dishes and were provided with moist food from time to time. The humidity was only controlled approximately, however, by adding drops of water to the substrate of filter-paper, so that his figures must be accepted with caution.

In conclusion, it is in our view misleading to speak of certain species of woodlice as living in dry surroundings. Until precise measurements of temperature and humidity in the micro-habitats of woodlice have been made, there can be no full understanding of the ecological situation; but instead of 'dry-living' and 'wet-living' species, it is probably nearer the truth to say that all species of woodlice are confined for the most part to micro-habitats where the saturation deficit is low and damp surfaces are available, but that they differ from each other in tolerance of suboptimal conditions, both as regards degree of adversity and period of exposure.

SUMMARY

1. Experiments were undertaken to determine the site of water uptake, and the conditions in which it occurs, in *Ligia oceanica*, *Oniscus asellus*, *Porcellio scaber* and *Armadillidium vulgare*.

2. Besides absorbing water with moist food, all four species were capable of active imbibition of water through both mouth and anus when a free water surface was available. In *Ligia oceanica*, imbibition occurred largely through the anus, in other species, through the mouth. Absorption through the anus was demonstrated by blocking the mouth by electro-cautery.

3. All species save *L. oceanica* could imbibe water through the mouth from a damp surface (plaster of Paris) sufficiently rapidly to counteract the effect of transpiration from the integument in air at *c.* 85 % R.H. No water was absorbed through the anus in these conditions.

4. The results help to resolve certain conflicting statements regarding the distribution of different species in the field. All species are for the most part 'wet living', but they differ as regards tolerance of suboptimal conditions.

This work was carried out while J. E. Spencer was in receipt of a grant from the Department of Scientific and Industrial Research.

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A NOTE ON THE PHARMACOLOGY OF THE DORSAL MUSCULATURE OF *PERIPATOPSIS*

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(*Received* 21 *January* 1954)

As far as they have been studied the muscles of the body wall of annelids have been found to be sensitive to acetylcholine. The dorsal musculature of *Hirudo medicinalis* L. responds by contraction to a low concentration of the drug (Minz, 1932); among the chaetopods a similar effect has been shown with the body wall of *Lumbricus terrestris* L., *Arenicola marina* L. (Wu, 1939) and *Branchiomma vesiculosum* (Montagu) (Nicol, 1952). In all cases the threshold of response is at dilutions of 10^{-4} to 10^{-5}. No such responses have generally been found with the sketetal muscles of the Crustacea. Bacq (1935) reports that the muscles of *Carcinus maenas* (Pennant) and *Lepas anatifera* L. do not respond to acetylcholine at a concentration of 10^{-4}; du Buy (1935) was unable to elicit any response to crystals of the drug from the muscles of *Cambarus* sp.; Katz (1936) found no response from the muscles of *Carcinus maenas* to acetylcholine at a concentration of 10^{-5}, but observed a 'moderate short contracture' to a concentration of 10^{-4};* Ellis, Thienes & Wiersma (1942) detected no response from the claw muscles of *Cambarus clarkii* Girard even at concentrations of 10^{-1}.

In view of the morphological similarities of the Onychophora to both annelids and arthropods, it appeared to be of interest to study the responses of the muscles of their body wall to acetylcholine.

Specimens of *Peripatopsis moseleyi* (Wood-Mason) were used. An animal was gently stroked on the dorsal surface until it had ceased to spit. The head and posterior end were then cut off and the viscera extruded by gentle pressure. Two lateral cuts were made just dorsal to the legs so as to separate a length of dorsal longitudinal muscle. The strip of muscle was ligatured at either end and mounted, with the inner surface uppermost, in a muscle chamber. The preparation was attached to a light isotonic lever. The muscle was kept moist by a drip of saline containing 6·0 g. sodium chloride and 0·16 g. calcium chloride per litre. The various drugs used were dissolved in this solution. Dilutions are expressed in grams per millilitre. Owing to the instability of acetylcholine in neutral solutions, all solutions employed were brought to a pH of 3·8 before use. This acidity did not appear to affect the response of the muscle to direct electrical stimulation. The relative scarcity of animals has limited the number of experiments which could be undertaken. With this technique the following observations on the effects of various drugs have been made:

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* Katz has given reasons for the belief that this response was due to a direct action of the drug upon the motor nerve (Bacq, 1937).

Acetylcholine. The strips of *Peripatopsis* muscle responded with a slow contraction which reached its maximum in 3–4 min. Relaxation was correspondingly slow. The threshold of response lay below a concentration of 3×10^{-6}. This sensitivity is of the same order as that which has been found in annelids.

Eserine. The muscle responds by a contraction to a concentration of 2×10^{-6}; there is no response to a concentration of $1{\cdot}4 \times 10^{-6}$.

Acetylcholine following eserine. If the muscle is first stimulated to contract with 3×10^{-6} acetylcholine solution, then washed in saline for about 10 min., then in eserine of a concentration of $1{\cdot}4 \times 10^{-6}$ for 3 min. and finally restimulated with acetylcholine, the second response is markedly greater than the first (Fig. 1). This effect has been demonstrated several times.

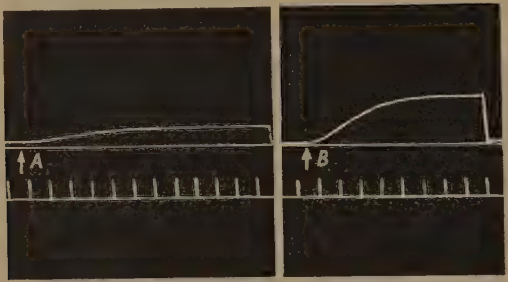

Fig. 1. Response of the dorsal longitudinal muscles of *Peripatopsis* to acetylcholine. At *A* the preparation was treated with 3×10^{-6} acetylcholine solution. The preparation was then washed, first with saline and then for three minutes with $1{\cdot}4 \times 10^{-6}$ eserine solution. At *B* the preparation was again treated with 3×10^{-6} acetylcholine solution. Time marker, 30 sec.

Atropine. This drug causes the muscle to contract at a concentration of $2{\cdot}5 \times 10^{-5}$, but not of $1{\cdot}2 \times 10^{-5}$. The response of the muscle to acetylcholine at a concentration of 5×10^{-5} is not modified by previous irrigation with atropine at a concentration of $1{\cdot}2 \times 10^{-5}$.

Curare. This drug causes no contraction of the muscle at a concentration of 5×10^{-5}, nor, at this concentration, does it modify the response of the muscle to acetylcholine at a concentration of 5×10^{-5}.

Adrenaline. No response was elicited from the muscle by solutions of adrenaline of a concentration of 10^{-4}.

It is clear that these results indicate that, pharmacologically, the musculature of the body wall of *Peripatopsis* has much in common with that of the annelids and differs from the skeletal musculature of the Crustacea in its responsiveness to acetylcholine. Tables 1 and 2 compare the results obtained by previous workers on annelid material with the present observations. The behaviour of *Peripatopsis* muscle is distinct from that of *Hirudo* for, as Bacq & Coppée (1937) have shown, the locomotor muscles of the latter are partially paralysed by curare. The musculature of *Peripatopsis* resembles that of *Lumbricus* and differs from that of *Arenicola*

Table 1. *Threshold of response of body-wall musculature to different drugs*

Drug	*Arenicola* (Wu, 1939)	*Branchiomma* (Nicol, 1952)	*Lumbricus* (Wu, 1939)	*Hirudo* (Minz, 1932)	*Peripatopsis* (present paper)
Acetylcholine	10^{-5}	10^{-5}	10^{-5}	10^{-5}	3×10^{-6}
Eserine	No response	No response	10^{-7}	—	2×10^{-6}
Atropine	—	No response	10^{-5}	5×10^{-5}	2×10^{-5}
Adrenaline	No clear response	No response	—	No response	No response

Table 2. *Effect of various drugs upon the response of body-wall musculature to acetylcholine*

Drug	*Arenicola* (Wu, 1939)	*Branchiomma* (Nicol, 1952)	*Lumbricus* (Wu, 1939)	*Hirudo* (Minz, 1932)	*Peripatopsis* (present paper)
Eserine	Threshold lowered	Potentiation	Threshold lowered	Threshold lowered	Potentiation
Atropine	Response depressed	No effect	Response depressed	No effect	No effect
Curare	—	No effect*	—	—	No effect

* This result refers to the effect of curare upon the response to indirect electrical stimulation and not to the effect upon the direct action of acetylcholine.

and *Branchiomma* in its direct responsiveness to eserine and atropine. The sensitiveness of the body-wall muscles of *Lumbricus* to curare does not appear to have been investigated, but we have observed that this drug does not modify the response to acetylcholine of the body-wall musculature of the earthworm *Microchaetus*.

Tiegs (1947) has recently concluded that the myriapod-insect line has arisen independently of the other major groups of arthropods from some ancient stock of Peripatus-like ancestors. If pharmacological behaviour has any systematic significance it is therefore relevant to inquire whether the skeletal muscles of the myriapods are sensitive to acetylcholine. We have observed the effect of the drug on the dorsal musculature of the chilopod *Cormocephalus multispinus* (Kraep), of the diplopod *Doratogonus setosus* (Vog.) and of the caterpillars of the moth *Gonometa pastica* Wlk. In no case did acetylcholine at a concentration of 10^{-4} stimulate the muscles to contract.

The fact that the Onychophora have smooth muscle is an annelidan character which contrasts with the characteristically striped muscle of the Arthropoda. The present experiments show that not only in their histology but also in their pharmacology onychophoran muscles are akin to those of the annelids and, within the present limits of our knowledge, more especially to those of the Oligochaeta.

SUMMARY

The muscles of the dorsal body wall of *Peripatopsis moseleyi* respond to acetylcholine at a concentration of 3×10^{-6}. This contraction is enhanced by previous treatment with eserine and it not inhibited by either atropine or curare. Adrenaline produces no contraction.

Within the limits of our present knowledge these muscles resemble, in their responses to drugs, those of the chaetopod annelids and differ from those of crustaceans, myriapods and insects.

Our thanks are due to Dr Michael Barton for the gift of curare and to Dr R. F. Lawrence and Mr Peter Metcalfe for identifications.

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STRUCTURE AND FUNCTION IN THE LOCOMOTORY SYSTEM OF THE DOGFISH EMBRYO. THE MYOGENIC STAGE OF MOVEMENT

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(*Received* 21 *January* 1954)

INTRODUCTION

The ultimate goal of a physiological analysis of animal behaviour must be to interpret actions, however complex, and whether instinctive or learned, in terms of the detailed structure and properties of the neuromuscular system. This is still so far from being achieved that the simplest type of behaviour of lower vertebrate embryos is not yet capable of such an interpretation.

Like many other workers, we have studied the behaviour-mechanism of locomotion, because the characteristic adult features of this pattern are well known and the stages through which they pass in development are usually recognizable. Though reference will be made from time to time to unpublished work on the trout and lamprey, the present account deals with the earliest movements of *Scyliorhinus caniculus* (L).

There are certain *structural features* in the lower vertebrate embryo which are significant. In early motile stages, the myotomes lie lateral and ventro-lateral to the notochord. Within each myotome, the myofibrils run parallel to the notochord throughout the length of the myotome, between its anterior and posterior vertical surfaces. The myofibrils may be grouped into a pile of horizontal syncytial muscle plates; in such cases the muscle plate would constitute the minimum unit of synchronously contracting tissue, though several may be innervated by a single motor nerve fibre. The three pairs of pre-otic myotomes never form part of the locomotor system, but all the post-otic myotomes developed at this period may do so, and may thus be grouped as 'trunk' myotomes. A similar lack of regional differentiation in the nervous system of the whole post-otic region makes it appropriate to refer to this as 'nerve cord' rather than 'spinal cord'; it includes part of the future hindbrain.

The earliest fully functioning neuromuscular system of the embryo possesses: ventral motor neurons whose axons make contact with some anterior somites, intra-medullary dorsal sensory neurons (Rohon-Beard neurons), and internuncial cells linking these sensory cells with contralateral motor cells. The sensory cells are in synaptic relation with one another longitudinally, but there are no long ascending tracts to the brain. Motor cells are similarly linked together by short longitudinal fibres, but in addition may be linked, by long descending fibres from co-ordinating neurons, into left and right neuromotor units.

The number of units involved in a neuromuscular system of this grade is remarkably small. Fewer than ten somites are involved in the first contractions; the most advanced of these somites will contain about a dozen differentiated muscle plates. The nervous structure is equally simple; we have established that the newly hatched lamprey embryo, which has been motile in the egg for several days, has about eight pairs of descending co-ordinating axons; corresponding to each functional somite there is only a single crossed internuncial neuron. In the trout embryo at the early motile stage there are one or two Rohon-Beard cells and about four motor neurons per somite.

The structure of the fully functional system in embryonic elasmobranchs will be dealt with in a later paper; in general it resembles that of other lower vertebrates except for a striking absence of large identifiable co-ordinating neurons.

The *development of behaviour patterns* in early motile stages of lower vertebrates has been studied extensively and is summarized in Hooker (1952). The conclusions of Coghill (1929), the most active pioneer in this field, may be briefly summarized in the following general terms. In the urodele *Amblystoma* the pattern of behaviour is at first total, i.e. exhibited by the entire available effector system. Separate items of behaviour are later individuated within this total pattern, until separate spinal reflexes may be elicited; throughout the process an integrated pattern of behaviour of the animal-as-a-whole is maintained. Coghill's views were clearly influenced by the 'Gestalt' school of behaviour. On the structural side, he considered that changes in the behaviour pattern were synchronous with, and caused by, changes in nervous structure: e.g. an already functional neuron might develop a new synaptic relation or a neuroblast might become a functional neuron in an already working sensori-motor arc (see Youngstrom, 1940). Coghill assumed that the integrated pattern of later behaviour was due to a successive development of higher centres in the brain. These views of Coghill have been challenged, but are at present generally accepted as applicable to lower vertebrates (see also Barron, 1941). Nevertheless, the study of behaviour in relation to nervous structure in elasmobranchs raises a number of problems in the discussion of his otherwise very plausible thesis.

Paton (1907, 1911) was the first to study this correlation in elasmobranch embryos; he claimed that there was an appreciable period in which somatic movements occurred before they were under nervous control, and that such myogenic movements were rhythmical. Wintrebert (1920–22) described the results of many years work on such embryos, and confirmed and greatly expanded Paton's account; his work has received surprisingly little attention in spite of its obvious importance. Wintrebert's outline (1920*d*) of the development of locomotion in the dogfish is based almost entirely on a very skilful analysis of the observed movements at closely spaced intervals in Balfour's (1878) stages G to K; these correspond to stages 15 to 24 inclusive of the Scammon (1911) scale. Briefly, he claims that the first movements originate within the separate myotome columns of both sides and are transmitted from myotome to myotome by direct excitation, i.e. without involving the mechanical pull of one on the next in the series. The two sides show regular but independent rhythms. Later the nerve cord takes over the initiation of excitation of the two separate units, the mode of conduction along the myotome bands remaining unchanged. Reciprocal inhibition and excitation within the cord then serves to co-ordinate the movements of the right and left sides and only when this third phase is over are true reflex circuits, involving the sensory neurons, brought into play. It is clearly important to substantiate and extend this account, not only by confirming Wintrebert's own observations, but also by correlating them as exactly as possible with the morphological pattern in the nervous system. The present study was commenced, in ignorance of Wintrebert's findings, some 15 years ago, but the intervention of the war years and the necessity for overcoming various problems in neurological technique (see Whiting, 1948; Harris & Peters, 1953), and for providing an accurately timed series of embryos (Harris, 1952), has only now made it practicable to provide a sufficiently critical account of the initial stage. The next, neurogenic, stage of locomotion will be dealt with in a later paper.

The rhythmical nature of the early body movements appears to be peculiar to elasmobranchs; it has not been reported in other fishes, and we can confirm from our own observations that it does not occur in the lamprey and trout embryos. Such a rhythm might originate in the muscles (myogenic excitation) or in the nervous system (neurogenic excitation) either from motor neurons or from more distant connexions; it might, thirdly, be a self-exciting reflex rhythm. Only a careful and detailed study of both the physiological and morphological features of the early movements can reveal the most probable pattern.

On the physiological side, the existence of a rhythmical contraction is both a hindrance and a help. It helps greatly in deciding whether experimental interference changes the process in any way, since quite small changes in frequency and phasing of a rhythmical contraction are easy to recognize. On the other hand, at a later stage it makes difficult the exact interpretation of the effect of stimulation since the timing of the stimulus in such cases is as critical as its intensity.

MATERIAL AND METHODS

The elasmobranch embryos on which the present work is based have been those of *Scyliorhinus caniculus*, obtained from the oviducts of dead fish consigned in a fresh condition from Ilfracombe. Even after the adults have been dead for at least 24 hr. such embryos show a surprisingly high percentage of successful development, hatching in about 9 months at 12° C. The time course of development has been described (Harris, 1952), and although material has never been so plentiful as one could wish, accurate knowledge of the rate of development has made it possible to obtain embryos at any required stage.

The critical stage with which we are concerned in this paper occurs after about 45 days' growth of the eggs at 12° C. (34 days at 15° C.). At this stage, development of the muscular and nervous systems is proceeding rapidly; the whole of the myogenic phase of embryonic movement occupies 2–3 days. Accurate morphological criteria are therefore essential. Fortunately we have found that the 'Normentafeln' of Scammon (1911), which were worked out on *Squalus acanthias* are an extremely accurate guide to the corresponding stages in *Scyliorhinus caniculus*, even to the extent of length of embryo and number of developed somites, characters which might well be expected to vary between squaloid and galeoid embryos. The stages of *S. caniculus* described by Balfour in his classical work on this embryo are too far apart (especially the stages F and G of Balfour) to be used here, and other descriptions depend on far fewer criteria than those given by Scammon.

In this paper the results of other investigators have, so far as possible, been expressed in terms of Scammon's stages of *Squalus acanthias* even where other species are concerned. The correspondence in all the early stages is sufficiently close for this to be done with no serious error.

The first embryonic movements occur during stage 16 of Scammon; the externally visible morphological criteria which we have used in establishing this stage are set out below:

Stage 15. Length about 3·3 mm.; 14–18 somites. One pair of well-defined gill-pouches visible by their transparency. Narrow anterior neuropore.

Stage 16. Length about 3·6 mm.; 18–21 somites. Two pairs of well-defined gill-pouches. Anterior neuropore minute or absent. More than half the length of embryo attached to yolk-sac. Heart visible as paired strands of cells. No pronephric rudiments visible. No depression in otic region; placode visible as epithelial thickening.

Stage 17. Length about 4·0 mm.; 21–25 somites. Less than half the embryo attached to yolk-sac. Anterior part of heart is median and tubular. Pronephric rudiments on three pairs of somites. Otic pit visible as a slight depression. Post-cloacal region now more than twice as long as deep.

The length and somite number increase slowly at first and are not reliable criteria until after stage 16. The numbering of the somites immediately following the otic capsule has provoked much discussion. We have followed the opinion of Goodrich (1918) rather than that of van Wijhe in this matter.

The nervous structure of the embryos was studied by the use of the following techniques: block-silver; vom Rath's platinic chloride; silver on the slide; the Sihler-Gad haematoxylin procedure; methylene blue.

A block-silver method was successfully adapted for use on older embryos, but was not satisfactory for younger stages. Vom Rath's and the Sihler-Gad methods gave little promise and were not pursued.

Silvering on the slide gave satisfactory results on all stages of embryo, but, for the youngest embryos, each slide required careful individual treatment. The procedure was the same as that used for the pro-ammocoete of *Lampetra* (Whiting, 1948). Both Bodian's Protargol and Holmes's silver nitrate techniques were effective after Nonidez's fixative and Lang's method for dehydration. Lang's alcohols contain *n*-butyl alcohol, as did the only successful fixative used on block-silver preparations. The youngest embryos needed especial care during reduction with oxalic acid; this was partly because they were too small for the detail to be visible with a low-power objective. Even after mounting the slides, it was sometimes necessary laboriously to return them to water for treatment with Farmer's solution. Thick sections (16μ) were usually impregnated better than thinner ones.

In stages 16 and 17 the silver methods showed up different types of neuroblast with varying success. Rohon-Beard cells were somewhat darker than other dorsal cells, but the short broad processes of their early stages of outgrowth were pale and difficult to define. Developing dorso-lateral neuroblasts were well differentiated, the axon and growth-cone being almost black, and the cell body much darker than surrounding cells. The whole extent of these cells was often contained within a single transverse section, but where it was necessary to trace the axon through a few sections, this could in most cases be done with precision.

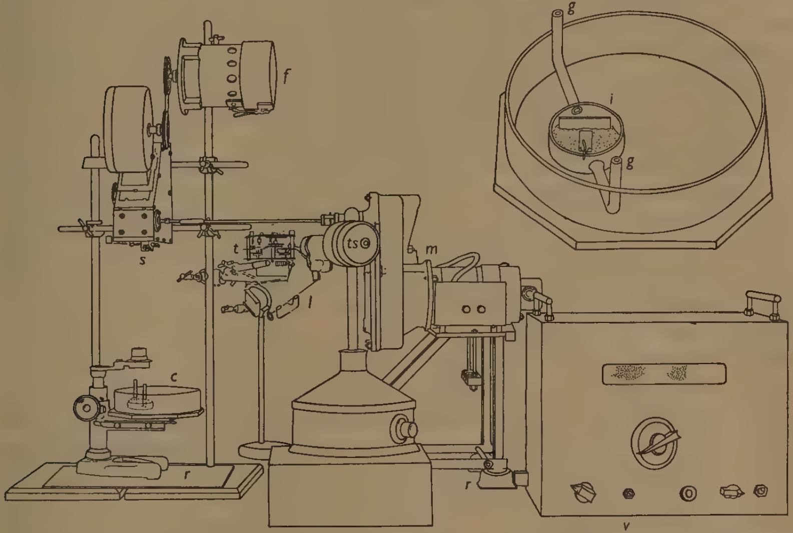

Fig. 1. The recording apparatus in its present form. A 35 mm. recording slit camera (*s*), is driven from a rotary convertor and gear train, (*m*), which is energized from an electronically controlled circuit of the velodyne type, (*v*). The take-up for the film is driven by a separate motor (*f*). (*t*) is a rotating mirror time-marker and (*ts*) its light source. The embryo is mounted in the special cell (*c*) on the stage of a simple dissecting microscope illuminated from the lamp (*l*). Rubber mats and antivibration mountings (*r*) serve to isolate the moving parts. All wiring is omitted for clarity. The cell is shown in greater detail in the inset. It contains an inner compartment (*i*), in which the embryo is mounted for photography; the outer compartment is used for preliminary operations on the egg. A small glass plate (not shown) can be placed over the top of the inner compartment, isolating it so that drug solutions can be perfused through the tubes (*gg*) during an experiment. A small vertical baffle plate prevents excessive disturbance of the embryo during such perfusion experiments.

Various methods of handling the embryo for physiological observations have been developed. Usually the egg-case was opened by cutting round one side of the shell; the open shell and its contents were then immersed in dogfish saline (Bialaszewicz, 1933) in a Petri dish, and the yolk and albumen were poured out on to the bottom of the dish. If the embryo had remained on the upper surface of the yolk it was a simple matter to cut the broad yolk-stalk from the extra-embryonic blastoderm and to free the tissues from yolk particles which might have adhered to it. The embryo was mounted on a base of paraffin wax by pinning it with entomological pins through the free edge of the yolk-stalk. A special plate-glass cell with a piece of clear glass slide let into the wax-covered bottom served to support the embryo so that the head and anterior trunk were free to move above the transparent area. Such an embryo will often continue its contractions for as long as 24-hr (see Fig. 1, inset).

The cell is mounted on the stage of a simple dissecting microscope, the mirror of which is illuminated by an ordinary microscope lamp; the silhouetted image of the head is projected by a single 40 mm. lens on to the 35 mm. film of a recording slit camera. Time-marking is accomplished by a rotating mirror system which illuminates the slit at regular intervals. Considerable difficulty has been encountered in making the film run smoothly at speeds as low as 1 mm./sec.; this is evident from the unevenness in lighting and the irregular position of the time-marks in the records which follow.

A second method of recording, by reflected light, has also been used with certain advantages. The egg-case is held under dogfish saline in a short specimen tube with the blunt (normally top) end of the egg-case uppermost. The top end is cut off and the shell trimmed down so that the embryo projects just above the level of the edge of the egg-case. Without damaging the blastoderm, a short length ($\frac{1}{2}$–1 mm.) of fine black human hair is thrust backwards through the closed medullary folds at the future midbrain roof into the neural canal, coming to lie along the axis of the main part of the nerve cord. This provides a black pointer which oscillates at the tip with the slightest movement of the head. The embryo is illuminated by a concentrated beam from a 48 W. microscope lamp, and the dark image of the pointer is recorded by reflected light on the film of the slit camera above. Using this method it is also possible to record wider excursions of the head. An automatic timing circuit, connected to the light sources and camera drive, was arranged to record for 10 min. every 2 hr. This method has given a limited success in records over a 12 hr. period; unfortunately the yolk eventually is forced up towards the surface so that the embryo is usually displaced after this time.

So far as possible, exact correlation of the recording with the structural analysis was achieved by studying the morphology of the actual embryos filmed or visually studied. Each embryo was anaesthetized in urethane immediately after the observations were completed; it was fixed either in Nonidez's fixative for the detailed study of the nervous system or in Susa for the study of general microscopic anatomy. Other fixatives were sometimes used for this latter purpose, but were less satisfactory. The successful silver staining of these very young stages is an uncertain matter but the nervous structures illustrated in the present paper are taken from embryos whose movements have been studied in detail.

THE EMBRYONIC MOVEMENTS

Previous work. In the youngest stages of *Pristiurus* and *Scyliorhinus* (3·5–4·0 mm. in length) Paton records rhythmical contractions at intervals of about 2 sec., interspaced with pauses of several seconds duration. These movements preceded the first contractions of the heart by 3–5 days. Cocaine had no effect on embryos between 4·5 and 6·5 mm. in length, either on trunk-muscle or heart contractions; at 7·0 mm. or larger, cocaine abolished the trunk contractions reversibly without affecting the heart rate.

Wintrebert, though he did less than justice to Paton's pioneer account, described the early embryonic movements in much greater detail. He stated that the period of myogenic (aneural) movements lasted from a stage equivalent to the end of stage 15 up to stage 19. The contraction of each column of myotomes was said to begin at a *myotome initiateur* which, at first located at about the fourth post-otic myotome, later moved back until by stage 18 or stage 19, the two pace-makers were in the middle third of the embryo; the wave of excitation passed forwards and backwards from the pace-maker somite.

The contractions on any one side succeeded one another at regular intervals, the frequency increasing with age and with any rise in temperature. Because of the slight difference in this frequency between the right and left sides a periodic limping movement—*boiterie*—was produced, thus:

$$L..R..L.R...L\,R....\frac{L}{R}....R\,L...R.L..R..L..R.$$

At the time when the two contractions coincided, the trunk shortened slightly without any lateral displacement and the head was raised in a 'shrugging' movement. The two rhythms continued unchanged by these varying phase-relationships until the end of the myogenic period. This cycle of movements continued with a slowly changing form of flexure until a point was reached at which simultaneous contractions failed to appear, the pattern of contractions altering slightly at the critical moment; Wintrebert (1920*d*, 1922*c*) regarded this stage as the beginning of nervous intervention.

Removal of the nerve cord during this myogenic period (Wintrebert, 1921*c*) resulted, after a long shock period, in the renewal and continuation of the rhythmic movements in an unaltered form. Removal of the nerve cord after the phase of nervous intervention had begun produced a reversion to the earlier type of rhythm. If the nerve cord were merely transected instead of removed, Wintrebert found that a contraction was transmitted along the myotome-band without being affected by the transection. He considered these observations as evidence that the contractions of myotomes during the first phase were initiated myogenically and transmitted myomyally, and that in the second phase they were still transmitted myomyally, but were initiated by the nervous system. Wintrebert (1922*b*) supported his account of *Scyliorhinus* by observations of similar behaviour in *Raia punctata* and *R. asterias*. Here the left and right rhythms during the first phase were of almost identical frequency and the periods of limping had a much longer duration. During such a period he found that light touch on an active muscle altered the rhythm on that side, so that the limping ended quickly and a regular alternation recommenced.

Our own observations. Movement in the embryo of *Scyliorhinus caniculus* begins at stage 16, when it is about 3·6 mm. in length and shows some 17 well-developed post-otic somites. At this stage the yolk-stalk connects to the yolk-sac a region of the trunk which is just over one-half of the total length of the embryo. The anterior third is free to move, and a very short length of the developing tail region projects behind the yolk-stalk.

The commencement of the earliest movements has only once been seen. In this single instance, the embryo, within a few minutes of its removal from the egg-case, gave a number of movements of the head to one side which settled down to a regular rhythm of unilateral contractions within the first 15 min. Before long, contractions of the opposite side occurred also and, less than an hour from its removal, a typical regular sequence of contractions of both sides was in progress. These early contractions occurred at intervals of 3 or 4 sec.; the flexure of the head on the trunk region was not more than 5°.

The time course of these early contractions is illustrated in Figs. 2 and 3*a* taken from an embryo late in stage 16. The outward swing of the head starts quite rapidly from the midline, its velocity falling as the angular displacement increases until the peak displacement is reached about one-third of a second after the start. The exponential character of the return suggests the existence of a viscous damping component, partly produced by the muscle and body tissues, but also by the resistance of the liquid medium itself; a passively displaced head shows a similar type of return to the midline.

The shortening of the myotome during a contraction can easily be seen in side view; it appears to be greatest at the level of the second to third post-otic somite, which is also the region of greatest inflexion when the embryo is viewed from above. As the contractions are small, it is not easy to see how many other myotomes are participating, but by the end of stage 16 it is clear that at least three somites are concerned; in later stages the number increases considerably.

The most obvious feature of these early contractions is their extreme regularity in timing and their constancy of amplitude on each side, together with a complete and striking independence of the right and left sides (see Fig. 3*a*, *b*). Thus in a typical record of some fifty contractions over a period of 40 sec., contractions to the right occurred at an average interval of 1·66 sec., with a standard deviation of individual observations of only 0·10 sec. In the same period the average interval between contractions to the left was 1·92 sec. (standard deviation 0·11 sec.).

If any further evidence were needed of the independence of the two sides, it is provided by the regular recurrence of phenomena which occur whenever the unequal intervals result in an almost simultaneous contraction of both sides. A brief excursion to one side starts at a speed which is indistinguishable from the normal but is cut off almost immediately in favour of a swing to the opposite side. When the contractions are simultaneous the effect is to produce a shortening of the body with little or no lateral flexure—the movement which Wintrebert has vividly called a 'shrugging movement'. Since the functional muscle is at this stage in the dorsal part of the myotome, the head in fact lifts during this shrugging movement.

Records of these almost coincidental right and left displacements provide further evidence regarding the time-course of the contraction itself. In these early stages there is no indication of a propagated contraction down the myotome band; the band appears to contract as a whole, which suggests that the conduction process along it is very much more rapid than the contraction process. The actual form of

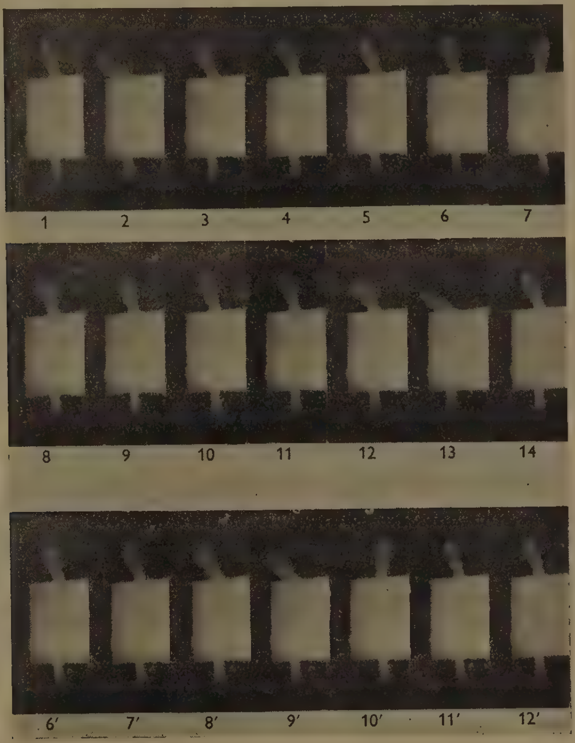

Fig. 2. Two sections 1–14 and 6′–12′, from a single film of the movement of an embryo of *S. caniculus* during the myogenic phase (stage 16–17). The head and tail regions are photographed against a black paper background; the mid-trunk region is still attached to the yolk-sac by a broad yolk-stalk. Photographs are shown at intervals of $\frac{1}{8}$ sec. The embryo is lying slightly on its left-hand side and the resting position is with the head slightly inclined to the left. From this position the first sequence of the film shows left, right and left contractions in frames 2, 7 and 13 respectively. The second short section of the film shows the right contraction, 10′, immediately following the left, 8′–9′; there is a preceding and following sequence of photographs 1′–5′ and 13′–17′ (not shown) during which there is no movement of the embryo.

the lateral displacement is 'ballistic' in type, and might suggest a very rapid twitch giving rise to a swing which dies away along a fully damped decay curve. But the shrug contraction, which produces no lateral swing, has a similar time-course except for the shorter duration of the exponential return. It follows that the muscle contraction is really a slow one and not only apparently so. This is borne out by

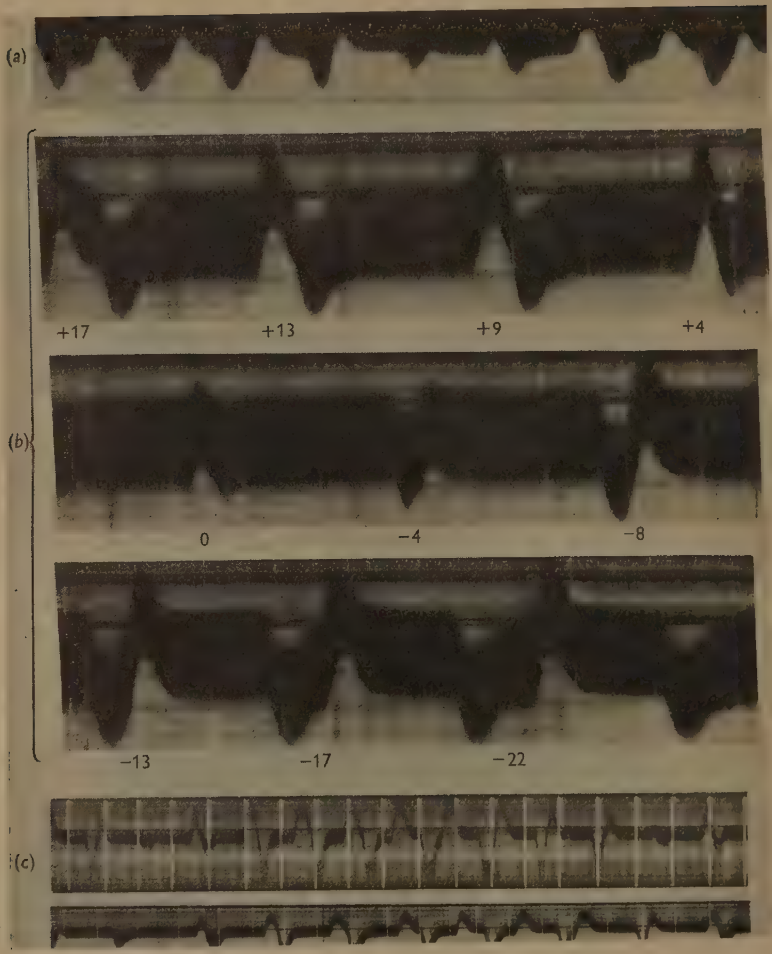

Fig. 3. (*a*) A record from the slit camera showing a sequence of movements of the head of an embryo of stage 16. Contractions to the right are shown downwards; to the left, upwards. The record reads from left to right. (*b*) A record from an embryo of stage 17 showing the form of the movements as the time interval varies between successive right and left flexures. This phase difference between any two successive contractions is shown below each movement; it is calculated on the assumption of a uniform frequency for each side. The form of the curves is to be compared with Fig. 2 in the text. (*c*) The effect of denervating an embryo (stage 20, for reasons given in the text). The upper record shows the movements of the intact embryo, the lower one those of the same embryo after complete removal of the nerve cord. The amplitude and frequency of movements are reduced after operation but the sequence and type of contractions is unaltered. The time marks are at intervals of 1·45 sec.

a study of the displacement curve in nearly simultaneous right and left contractions. Fig 4 shows that the early part of this compound displacement curve is quite accurately represented by the algebraic sum of the two separate displacements, taking into account the appropriate phase difference. This can only mean that during this period each position is in fact an equilibrium one, and that the displacement-time curve of each side represents to a first approximation a true shortening-time curve of the myotome. The agreement between the observed and calculated curves does not completely hold for the later part, where at least one of the muscle bands is relaxing.

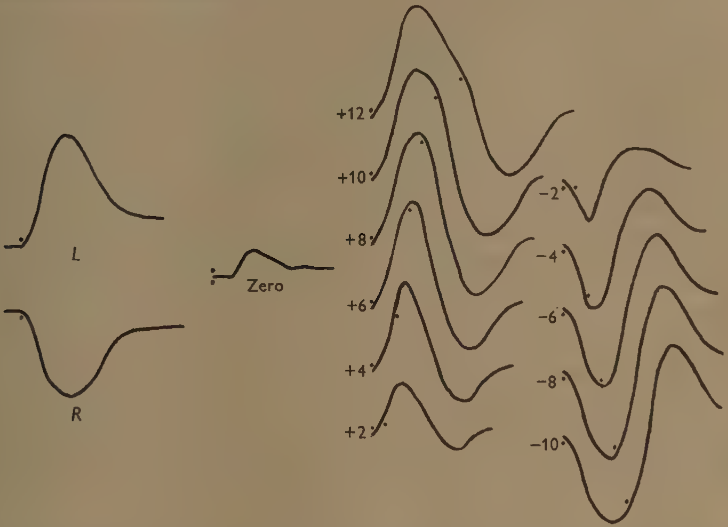

Fig. 4. The calculated displacement curves of the embryo photographed in Fig. 3 (*b*) for a series of time-intervals between the right and left contractions. Each curve is the algebraic sum of the two isolated curves *R* and *L* with a phase difference in arbitrary units indicated on each curve. The time scale used is identical with that in Fig. 3 (*b*); the similarity of the *calculated displacements* in Fig. 4 above with the observed ones in Fig. 3 (*b*) shows that the right and left contractions are completely independent.

The embryo in stages 16 and 17 is quite insensitive to any form of light tactile stimulation or to restraint, which do not appear to alter the pattern or frequency of the rhythm. With a suitable needle mounted in the de Fonbrune micromanipulator it has been found that extensive damage to the tissues, so long as it does not involve the actively contracting myotomes, also has no effect. It is even possible to tear the ventral portion of the third post-otic and neighbouring myotomes without destroying the sequence of movements; there is no doubt that these myotomes are actively contracting during the movements. Partial damage to the dorsal region of these myotomes is however immediately effective; usually the movement stops on that side for a period, but it is resumed within 15 min.

The embryonic contractions can be abolished in a dogfish saline containing 2% of urethane. In a film recorded throughout such an experiment, the frequency of contractions gradually diminished, though at a different rate on the two sides. Throughout the process the amplitude of the contractions was quite unchanged until they finally ceased; it is clear that the pace-maker mechanism was slowed down while the contraction process itself was unaffected.

Cutting off the head, even as far back as the first post-otic somite, stopped the movements for only a short period. The stoppage was evidently similar to that produced by damaging the functional region of an active myotome; within 1–15 min. the embryo resumed its normal pattern of movements.

A crucial experiment in establishing the myogenic nature of the rhythm is of course the complete denervation of the embryo. We have repeated and confirmed the results reported by Wintrebert (1921*c*). The operation is not easy to carry out in the youngest motile stages, owing to the small size and fragile tissues of the embryo; the recording of movements from such operated embryos is attended with considerable difficulty. We have done such operations on stage 17, but the records were not suitable for reproduction; Fig. 3*c* has therefore been recorded from an older embryo (stage 20) in which functional neuromuscular connexions had been established. In all such experiments, the embryo was dissected away from the yolk-sac, mounted on a glass platform and its movements were recorded in the usual way. It was then transferred to a second dish containing dogfish saline, and denervated. A horizontal cut was made in one side of the animal at the level of the dorsal surface of the notochord. The cut, started in the mid-trunk region, was extended forwards and backwards, freeing the nerve cord completely from the notochord over the whole length of the animal with the exception of the tip of the tail and the head. The posterior end of the freed nerve cord was transected and the cord lifted in a pair of fine forceps and peeled forwards out of the embryo; the brain was freed from the pharyngeal tissues as the separation was carried forward and the most anterior fore- and mid-brain region was removed as a whole. The few remaining pieces of nerve cord were dissected away from the notochord until the latter showed its complete unbroken dorsal surface. Finally the unoperated part of the tail was cut off.

Since this operation necessarily cuts the dorsal portions of all the anterior myotomes, it is not surprising that for a short time after the operation the embryo remains apparently motionless, though this may well represent an irregular but continued 'fibrillation' of the damaged muscles. Nevertheless, within 2–30 min. of the completion of the operation, every such operated embryo (of six in all) has recommenced rhythmical contractions first of one side and later of both sides, as shown in the record of Fig. 3*c*. The frequency of the contractions is usually somewhat slower than in the intact embryo, but this is scarcely surprising in view of the extensive damage involved.

A single experiment has taken this study one step further. An embryo at stage 17 was cut in half at the level of the third post-otic somite. As usual under such circumstances, within 5 or 10 min. the stumps of both ends were seen to give regular rhythmical right and left contractions, the frequency in the head stump being slightly faster than that of the muscle bands in the trunk portion. In this case the frequency was approximately ten contractions in 25 sec. A small piece of the fourth myotome on one side was excised from the trunk portion, and mounted in saline solution under a cover-slip. The fragment remained completely motionless for nearly half an hour, when a portion of it was seen to begin regular rhythmical contractions at the rate of 10 in 30 sec. These persisted for 15 min. until the preparation finally disintegrated.

Though it is proposed to leave to a later paper the description of studies on the embryo when nerve-muscle contact is fully established, it is not out of place here to point out that rhythmical contractions of myotome bands in a denervated embryo occur long after there is physiological and morphological evidence that the nervous system is participating in the movements of the intact animal. We have seen and recorded such movements up to stage 21; by stage 23, though the muscle bands still give co-ordinated contractions in the denervated preparation, the contractions are no longer rhythmical but occur at irregular intervals. By this time, either the muscle pace-maker has ceased to function rhythmically, or the excitation threshold of the muscle plates has risen so that they can only be effectively discharged through motor end-plates on the muscles themselves. Myomyal conduction is still possible, as in earlier stages, but the myogenic rhythm has disappeared.

THE NEUROMUSCULAR STRUCTURE

Previous work. In view of the very rapid development of the nervous system at this critical stage, a study of the relevant literature must distinguish the motor neurons from other cells with processes in the ventro-lateral region of the cord; it must take into account the time and mode of establishment of the first contact between the motor neuron fibres and the muscle.

The accounts of Rohon-Beard cells by Beard (1892, 1896), Studnicka (1895) and Paton (1907, 1911) as well as those of Neal (1914) all refer to stage 20 or later; in studies on internuncial cells Lenhossek (1892) and Retzius (1895) deal with much older embryos, as also do Neal's illustrations of dorso-lateral neurons. The early history of both these cell types is virtually unknown, but unless the reflex arc is completely established before movement begins the only relevant facts are those concerning the motor neurons.

Paton (1911) described motor neuroblasts with peripheral fibres reaching to the myotome in 5 mm. *Torpedo* and 6 mm. *Scyliorhinus* (stage 20/21), but his figures do not give much information.

Neal (1914) gave satisfactory illustrations of young motor neuroblasts at the cloacal level of the body in stage 21 of *Squalus* embryos. In a careful account he showed the steps by which the amoeboid growth-cone at the end of the axon grows ventro-laterally from the cord for the short distance to the myotome, where it turns up or down, adhering closely to the medial surface of the myotome. Neal stated: 'Somatic motor nerves make their first appearance in embryos of *Squalus* *acanthias* of about 4·5 mm. and approximately 30 somites. With their advent begins the rhythmic bending of the embryo. The first pair of somatic motor nerves to appear are those of van Wijhe's seventh somites, the first somites differentiated, and those which form the first permanent myotomes.' This places the contact of motor nerve and myotomes at stage 18. The pair of somites to which Neal refers is the third post-otic pair (cf. Johnston, 1911, Goodrich 1918).

The paper of Dart & Shellshear (1921) was an attempt to demonstrate the origin of motor neuroblasts from myotomes, and is of interest only because their Fig. 2 suggests that axons may have grown from the cord to the myotome at some level of a 4 mm. *Squalus*, which would place this event at about stage 17 if the embryo was measured before fixation.

Neal (1921) confirmed his previous account with preparations made by the Paton-Bielschowsky technique, and added some details. Motor cells, differentially impregnated even before a peripheral process has formed, are shown in ventro-lateral positions in the cord. The inner ends of the cells are usually still in contact with the neural canal; the long axis lies radially, and the cells end distally in the incipient neuraxon. In its further growth, the axon evidently meets a slight obstruction at the outer limiting membrane of the spinal cord, for it may turn tangentially here for a short distance before continuing radially towards the myotome. As before, no outgrowth of motor axons from the cord was observed before stage 18. There is no account of embryos of stage 16 which suggests that any neuroblasts have at this time developed a peripheral process.

It would therefore appear from the literature that no connexion has been established between nervous system and muscle or skin before stage 18 (possibly 17), in dogfish embryos. The first motor axons reach the myotome at stage 18 of *Squalus*, possibly later in *Scyliorhinus*.

The structure of the contractile tissue in *Scyliorhinus* has been clearly described by Balfour (1878). He stated that those cells of the inner layer of the myotome which were opposite the dorsal two-thirds of the notochord acquired a peculiar angular appearance in transverse section, and in horizontal section were seen to become elongated longitudinally to extend the whole length of a myotome as 'typical spindle-shaped embryonic muscle-cells, each with a large nucleus'. By stages 16 and 17 (Balfour's stage H), this small portion had become 'differentiated into a distinct band of longitudinal striated muscles; these almost at once become functional...' (i.e. by stages 18–20; Balfour's stage I). He considered the band as a precocious 'special formation'.

Balfour's figures of horizontal sections of an embryo of this age indicate that the spindle-shaped myoblasts of these bands tend to overlap each other longitudinally, crossing the boundary between one myotome and the next, in sharp contrast to the remaining cells of the myotome. This condition of overlap is transitory; it has been noted by Te Winkel (1948).

Thus the first myoblasts are contractile before any muscle-plates have been formed. But in many elasmobranchs these are in any case incompletely developed (Brachet, 1935).

Our own observations. In embryos at stage 15, the nerve cord is smoothly rounded. The only break to its cylindrical outline is the neural crest which is beginning to grow outwards and ventrally at the level of the future trigeminal ganglion. No axonic processes have been observed in the nerve cord; even the most advanced neuroblasts are little more than pear-shaped in outline. In the more anterior of the post-otic somites, that part of the inner wall of the myotome which is lateral to the notochord consists of closely packed spindle-shaped myoblasts arranged with the long axes lying longitudinally. While the rest of each myotome is sharply defined from its neighbour, these myoblasts are almost in contact with those of adjacent myotomes. Some cells of the inner wall have extended elongated processes on to the notochord, which already has a thin collagenous sheath. Similar processes extend from more dorsal cells of these myotomes up to the ventral and ventrolateral limit of the nerve cord, where they appear to come into direct contact with the adjacent neuroblasts.

In embryos at the beginning of stage 16, the degree of nervous development is indicated in Fig. 5, in an embryo which had begun to contract rhythmically. Development is most advanced at the level of the future otic pit, and is in general progressively less at more caudal levels. The anterior region behind the otic pit will of course become brain and not spinal cord, but since the myotomes of this region have not yet become reduced, all post-otic levels must be considered. No axons of motor neuroblasts were found, although some ventral cells were larger than the rest and were more darkly stained. Over a considerable length of the cord, dorsal cells have developed a short process which extends in a lateral direction just beneath the surface of the nerve cord. Lateral to these cells a number of neuroblasts have developed a longer axon, which is ventrally directed. These axons run circumferentially in the marginal layer of the cord, and are therefore even longer than they appear in the figure, where they are projected on to the median plane. Neither dendritic nor peripheral processes were observed in any neuroblasts. There is a marked tendency for the more advanced neuroblasts to occur in pairs, so that where a large neuroblast occurs on one side, there will usually be found a cell of similar form in the corresponding position on the opposite side.

The myoblasts near the notochord are now very elongated and overlap those of adjacent somites more than before. In a few of the anterior somites, especially in the third post-otic somite, myofibrils with clearly visible cross-striation can be seen in these myoblasts. The total number of such myoblasts is small, so that the development of cross-striation has probably been observed at its commencement. The degree of muscle development is indicated in Fig. 5.

In transverse sections the cytoplasm of the myoblast now appears to extend right across the inner wall of the myocoel, which suggests that the myoblasts are becoming arranged one above the other, in the form of incipient muscle-plates. Well-developed processes extend from the myotome into contact with the notochord and nerve cord; these attachments are more numerous than in the previous stage. Mesenchyme cells, with large amoeboid processes, occur between the somite and the pharyngeal endoderm, and in the channel between notochord, somite and endoderm. These general relations are illustrated in Fig. 6.

Less than 2 days later, at the end of stage 16, considerable further development is found. A three-dimensional model of the immediately post-otic region of the nervous system was made, showing the extent of some of the more prominent neuroblasts (see Fig. 7). No Rohon-Beard neuroblasts have been found with a process reaching skin or myotome. No cell bodies in the ventral part of the cord have a peripheral process at any level of the body in any embryo of this stage which has been examined, but more presumptive motor cells can now be found.

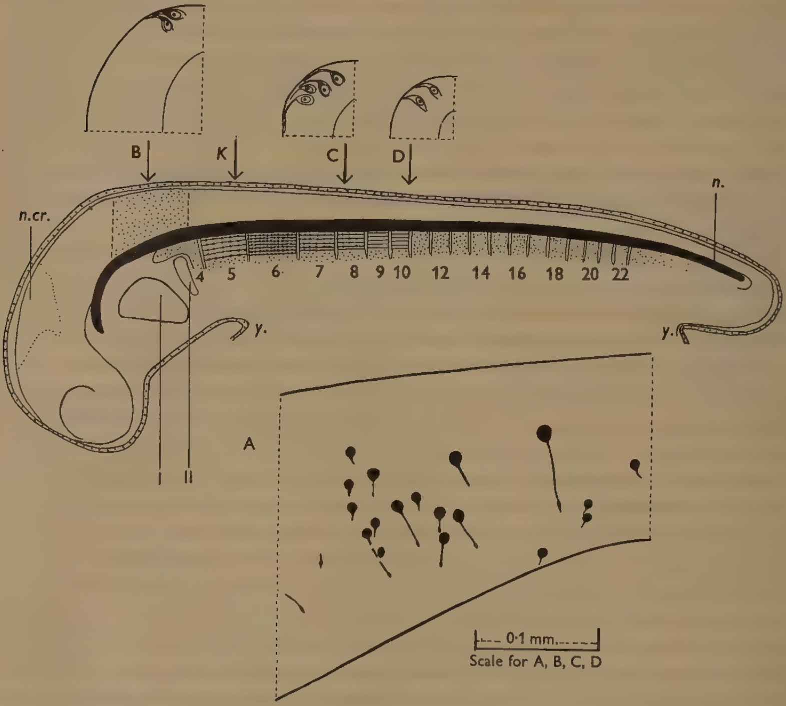

Fig. 5. Diagram of the central nervous system of an embryo at the beginning of stage 16, reconstructed from transverse sections. The embryo was about 3·3 mm. in length (fixed length 3·1 mm.), with 19 post-otic somites. The myotomes are drawn from sagittal sections and a whole mount of two similar embryos; the degree of development of myofibrils is indicated diagrammatically. *Inset A* represents the area of nerve cord stippled in the main figure; this region includes almost all the more prominent neuroblasts in the nerve cord at this stage. *Insets B, C and D* are quadrants of transverse sections of the cord at the levels indicated, and are drawn to the same scale as A; they show the degree of development of dorsal cells. *n.cr.* neural crest; *n.* notochord; *y. y.* limits of yolk stalk. I, hyoidean pouch; II, first branchial pouch.

The prominent dorso-lateral cells with a ventrally directed process have continued the ventral extension of this process against the limiting membrane. The course of these fibres has brought them close to the myotome, for the upper part of the medial surface of each myotome is concave and close to the neural tube (compare Figs. 6 and 7). By the end of stage 16, only a few hours after movement has commenced, it is therefore possible that a few lateral neuroblasts are in contact with cells of at least one post-otic myotome, by way of the axonic growth-cone and the dorsal processes of cells in the myotome. But these neurons are, as we shall show in a later paper, internuncial cells; the motor cells have not yet developed any process, and there is still at all levels a series of gaps in any potential sensori-motor arc. On the other hand, a structural pathway (the myotome band) exists for myomyal transmission of contraction between functional myoblasts of different somites.

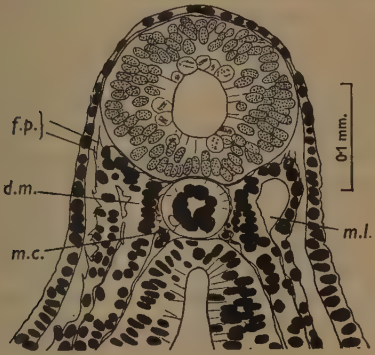

Fig. 6

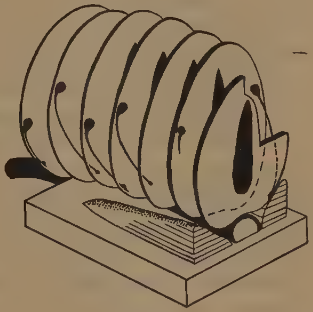

Fig. 7

Fig. 6. Transverse section of an embryo at stage 16 (20 somites), at level *K* of figure 5. Only the nuclei of the cells are shown. *f.p.* fibrillar processes from myotome; *d.m.* region of developing myoblasts; *m.c.* mesenchyme cells; *ml.* myocoel.

Fig. 7. An isometric reconstruction of the nerve cord and associated structures over the same region as inset A of Fig. 5, but from an embryo, at the end of stage 16, with 21 post-otic somites. The figure shows the further growth of the most developed neuroblasts; it is viewed from the posterior aspect; the notochord is shown black, and the upper portion of the fourth (first post-otic) myotome is shaded.

DISCUSSION

The results described here show in the first place that the left and right myotome-bands of the embryo dogfish function as a pair of rhythmically contracting units, each acting at this time independently of the other. In a system such as is found in these myotome-bands we must consider the various possibilities for both the origin and the transmission of the excitatory process leading up to the contraction of the individual myoblast.

A mechanism must exist for the *transmission* of the excitation from myoblast to myoblast within the myotome and also from myotome to myotome along the whole of the contractile region of one side, as well as for the *initiation* of this excitation in some centre of rhythmic discharge—a pace-maker either in contact with or a part of the myotome-band.

The results show that the nervous system plays no part in the mechanism for transmission of excitation, and indicate without reasonable doubt that initiation is also non-nervous. Transmission can be effected over the length of the contractile region after the nerve cord has been removed, and neither sensory, internuncial, nor motor neuroblasts have developed an axon at the relevant time and place. The character and form of the contractions—their remarkable constancy in amplitude and location—all support the view that we are dealing with a simple all-or-none process, comparable with the situation which exists in the vertebrate heart even before its innervation.

Regarding initiation, the physiological evidence is equally strong: rhythmical contraction is recommenced in the absence of the nerve cord; the initiation is a potential character of any contracting myotome since, when the myotome band is transected, each group of the contractile myotomes separated in this way sets up rhythmical contraction on its own.

The morphological evidence that the nervous system cannot be involved in the initiation is more complex. Over the short period between stages 15 and 17 of Scammon's embryonic series, the differentiation of neurons within the central nervous system is proceeding very rapidly. Many of the neuroblasts have during this time produced a single, long process, probably axonic in nature, which shows already a capacity for taking up silver and methylene blue. This process runs out towards, and then follows, the curve of the external border of the nerve cord in the region of the embryonic hindbrain; the expanded terminal growth-cone does not appear in any case to have passed beyond the limits of the cord. But at this position some of these neuroblasts are closely adherent to the surface of the cord, and the cord is at this level in contact with the most dorsal part of the myotome, so that it is not easy to say on morphological grounds alone that a structural neuromuscular contact has not been established. But there are excellent grounds for considering that a functional relationship has not yet been achieved.

Thus the most important reason for eliminating the nervous system entirely from consideration as the initiator of contraction at this stage of dogfish development is the experiment of Wintrebert—which we have confirmed—that rhythmical contraction occurs after the nervous system has been removed, and retains the same characteristics.

Anatomical reasons for reaching the same conclusion require careful analysis because they depend on negative evidence. Such reasons have been adduced by Paton on what can now be seen to be inadequate grounds, since the nervous system is appreciably more advanced at this stage than he and other previous workers supposed. Our own evidence is:

(i) The only neuroblasts which may be in contact with any myotome are not developed far enough to reach the somites when movement first begins (Fig. 5), although this degree of development is achieved soon afterwards (Fig. 7). This would be at least 24 hr after the first rhythmic movements.

(ii) The part of the myotome which is close to the growth-cone of these neuroblasts is the dorso-lateral edge, while it is the medial part next to the notochord which contains striated myoblasts.

(iii) The actual myotome which is in such close proximity to the neuroblast growth-cones is the first post-otic myotome in which no striated myoblasts have been found at this stage.

(iv) The neuroblasts concerned are not motor neuroblasts, which are developed later; they are probably future internuncial cells.

(v) Functional relationship between nerve and muscle might require motor end-plates, of which there is no trace.

The first reason, although depending on negative evidence and providing a very small time-margin—less than 1% of the period of embryonic development—appears to us to be based on sufficient evidence to be accepted as valid. Little weight can be put on the second and third arguments, on the other hand, because myoblasts which are near the nervous system but not yet functional might still be capable of transmitting excitation from a nervous initiator; furthermore, in the development of myoblasts in tissue culture, contractility has been found to precede the development of cross-striations, so that the myoblasts under consideration may be already functional. Equally, small reliance can be put on the fourth and fifth lines of approach; several examples could be given of neuroblasts changing function and losing old or gaining new synaptic relations after the time in ontogeny at which they first became functional. Motor end-plates are probably not essential structures for the transmission of excitation even at a stage which is known to be neurogenic; we have evidence for this statement in both the dogfish and the lamprey, and Speidel (1938) came to the same conclusion in his observations on regeneration of the motor axons in the tails of anuran larvae. Arguments (ii) to (iv) taken together may, however, properly be considered as supporting evidence adding to the cogency of (i).

It is therefore concluded not only that rhythmical contraction can be carried on without nervous initiation, but also that there is strong ground for thinking that nervous initiation is impossible at this stage. We must therefore consider the myotome bands as a pair of independent, rhythmically self-excitable motor units, with no means of excitation external to the myotome itself. Similar phenomena of rhythmical excitation and all-or-nothing contraction occur in many tissues in other animals. The most familiar examples are those of the heart and gut musculature, the first undoubtedly myogenic in the early embryonic condition, since rhythmical contractions in this organ begin before any of its later nervous connexions are established. The origin of excitation in the smooth muscle of the gut is also intrinsic to the muscle itself, though the co-ordination of peristaltic contractions is a nervous phenomenon.

The literature on the myogenic nature of the heart beat is extensive and comparatively well known; only in some arthropods is the origin of the beat in the adult to be found in the nervous system (e.g. *Limulus*, in Heinbecker, 1936), and even here there is ample evidence that the early embryonic heart-beat is myogenic

(Carlson & Meek, 1908). In molluscs and vertebrates the beat remains myogenic throughout life; although the rate of functioning of the non-nervous pace-maker is itself under nervous control, there is no synchronization of the nervous discharge with that of the heart contraction, as in *Limulus*. In such cases the action of drugs like acetylcholine and adrenaline can simulate completely the effect of stimulation by the nerves.

The observations of Goss (1942, 1952) on the heart of the embryo rat are of interest here. He found that this heart is rhythmically contractile while it is still in the form of two independent right and left parts, and that the contractile areas of the two sides then extend in the myocardial layer until they meet, when one side, usually the left with its faster rhythm, comes to set the pace for both sides so that a co-ordinated beat is developed in the single median heart. Since there is no nerve supply during this stage of development, the co-ordination of the beat is evidently a simple example of myomyal conduction from a single dominant pace-maker, which replaces two independent pace-makers acting on two separate contractile elements.

Rhythmical contraction appears to be an intrinsic possibility for many types of cell particularly in the early, less differentiated stage of development. Lumsden & Pomerat (1951) have recorded rhythmical contractions in cells, identified as oligodendroglia, in tissue-culture preparations of the brain of the rat. Lewis (1915) has described rhythmical contractions in myoblasts as well as in muscle fibres growing in tissue cultures of skeletal muscle of the chick. Particular myoblasts were found to have constant individual rates of rhythmic contraction. Yamamoto (1928, 1931, 1936) described rhythmical contractions spreading over the surface of the fertilized egg of the cyprinodont fish *Oryzias latipes*, similar to those described in the early development of many lower vertebrates and associated with changes in electrical impedance of fish eggs: see also Kuhl (1939) and Rothschild (1947). The contractions continue in *Oryzias* up to stages when the embryo is eyed, has melanophores and a circulating blood supply, and when the contracting surface is formed by the layer of future epidermal cells over the yolk-sac. The contractions are very slow; each commences at the embryo and spreads horizontally along a 'latitude' over the surface of the ectoderm until it reaches the opposite side of the embryo. In a contracting part of the ectoderm, the cells have a wavy outline and a reduced area, quite distinct from their appearance in a non-contracting area. At first the contractions originating on the two sides bear no temporal relation to each other, but later come to alternate with each other in a fairly regular manner. The alternation appears to arise from a reflexion of the wave of contraction when it arrives at the opposite side.

The most striking parallel to the myogenic contractions of the embryo dogfish is found in the rhythmical pulsation of the lymph hearts of the Amphibia (Pratt & Reid, 1932; Reid, 1937). These structures are derived from skeletal muscle, and their homology with such muscle is confirmed by the fact that the lymph-heart beat is neurogenically initiated: the anterior and posterior lymph hearts of the intact toad beat at the same frequency through a simple spinal reflex mechanism. Yet when denervated and transplanted under the tongue of the animal they develop a new myogenic rhythm of their own. Acetylcholine accelerates this rhythm in the transplanted denervated heart, while it causes reflex inhibition in the intact heart, as in the normal systemic heart (Reid, in Prosser, 1950). In embryonic systemic hearts before innervation, acetylcholine is said to have no effect. Our experiments on the myogenic rhythmical contraction of the dogfish myotome have been limited in number and are not yet conclusive, but they appear to suggest an acceleration of the rhythm in presence of acetylcholine.

Turning from biological analogies to studies of the dogfish embryo, we find that Paton was the first to state that early movements of embryonic elasmobranchs were myogenic and rhythmical. He regarded movement as myogenic on the basis of somewhat inadequate negative neurological evidence, already discussed, and as a deduction from the fact that cocaine has no effect on such early movements. At a length of 7 mm., when sensory nerves were found to reach the epidermis, he claimed that cocaine inhibited the contractions of *Scyliorhinus*. His account of the rhythm was also not very conclusive: 'Several of these movements, more or less rhythmical in character, occur at intervals of one or two seconds and are followed by a pause of several seconds and then the whole cycle begins again.' (Paton 1907, on *Pristiurus* 3·5 mm. embryos.) He failed to notice that the rhythm was in fact continuous; the regular short interruptions recorded by him are obviously the periodic pauses due to the nearly simultaneous contraction of the two sides, and it was left to Wintrebert to establish the significance of this feature. Both Paton and Wintrebert described the lack of sensitivity to external stimulation during this myogenic phase; only direct damage to the contracting myotomes had any effect, though Wintrebert describes, for a later stage of development, an effect on the rate of the (anterior) pacemaker produced by pricking one of the posterior somites.

Wintrebert is extremely precise in locating the pace-maker in the earliest embryos in the fourth post-otic myotome; from this position he claims that it moves backwards attaining the tenth post-otic myotome in stage 18. Whilst we should agree in general with the observations made by him on the form of the wave of contraction, without a very careful and detailed study of cinematograph films of the movement and an accurate knowledge of the mechanical restraint to displacement offered by the yolk-stalk, the viscous resistance of the medium and the internal elasticity of the notochord and other tissues, we doubt whether it is possible to make such a definite deduction.

The problem of the transmission of the contraction wave over the myotome band warrants a good deal of further attention. In the dogfish muscle, as in many other recorded examples, cross-striation appears after the myoblast is capable of contraction, and both rhythmical excitation and conduction are therefore not dependent upon the development of cross striations. Older embryonic muscle forms muscle-plates in most lower vertebrates, but at the stages described in this paper the myoblasts are still spindle-shaped, with their nuclei lying to one side of the contractile portion, rather like the contractile cells of *Hydra* or *Ascaris*. The muscle-plate stage (see, for example, Brachet, 1935), corresponds perhaps to the myotube in the mammal; in both cases the final stage is the production of a muscle fibre in the ordinary sense of the term.

Transmission of the excitation from myoblast to myoblast is obviously mediated by some mechanism involving surface contact between the two; it would therefore be quite comparable to the slow transmission of the contraction-wave over the blastoderm of *Oryzias*. The same mechanism would provide for transmission from myotome to myotome, as Te Winkel (1948) claimed; we have fully confirmed from longitudinal sections of these early embryos that there is an interdigitation of several of the myoblasts across the plane of the future myocomma. At a later stage these overlapping myoblasts are not found, but by this time nervous co-ordination of the myotomic contractions is adequate to account for the whole of the excitation and transmission process.

In summarizing the conclusions to which this present study has led us, it is necessary to point out in what respect they extend as well as merely confirm those of Wintrebert. The fact that the early movements are rhythmical and of constant amplitude and that the rhythm of the two sides is independent and unaffected by external stimulation does not exclude the participation of motor nerve-cells in the excitation process. Wintrebert has himself suggested that the first sign of the participation of the nervous system is a change in the character of the rhythm—not the interpolation of individual extra contractions (or inhibition of individual contractions) in the normal cycle. One tends only too easily to think of the effect of neurogenic excitation in terms of the adult motor unit—a single discrete response to a single nerve stimulus. Yet there is ample evidence in the adult sense-organ of a system in which the non-rhythmical build-up of some depolarizing process produces a rhythmical discharge of a sensory fibre. Similar relationship of a rise in electrotonic potential in the ventral root of the frog to the periodic spike discharge (Barron & Matthews 1938), as well as the repetitive discharge in cat muscle, when a single nerve impulse excites an eserinized end-plate (Brown, Dale & Feldberg, 1936), shows that the phenomenon is of general occurrence in excitation processes even on the motor side of the reflex arc; the normal discrete single responses of a motor unit are in part a function of the rate of accommodation of the excited element and in part a function of the peculiar conditions established by the existence of an elaborate motor end-plate with its high content of cholinesterase. The early dogfish embryo possesses no motor end-plates; its sensitivity to acetylcholine suggests that the cholinesterase mechanism is poorly developed in the myogenic phase,—we cannot therefore exclude the possibility that rhythmic muscle response may occur in the early embryo as a result of nerve excitation which is not itself synchronized with the muscle rhythm.

The morphological evidence provided here is therefore of considerable importance. The absence of any nerve-muscle contact in the earliest stages argues strongly against a neurogenic excitation process; while such negative evidence is exceedingly difficult to establish, the whole pattern and rate of development of the spinal neurons confirms the reliability of this conclusion.

Removal of the nerve cord, as in Wintrebert's own experiments, has not eliminated the rhythmical contractions. Our experiments were carried out in a dogfish saline solution; those of Wintrebert were done in sea water. He realized the possibility that sea water might be an 'unsuitable' physiological medium (Wintrebert, 1921*c*), and in fact claimed that contraction was only resumed after the wound made on removing the nerve cord had begun to heal over, but he did not appreciate the possibility that the ensuing rhythmical contractions might themselves be a consequence of abnormal ionic balance. The fact that our animals recovered their characteristic rhythm in dogfish saline within a few minutes of the operation suggests that the excitation mechanism was substantially unchanged. The great similarity in the frequency recorded in isolated parts of the myotome band, whether by decapitation of the embryo or even by isolation of an individual myotome seems to provide conclusive support for the view that in these early stages the excitation process, as well as the conduction from myoblast to myoblast and myotome to myotome, is intrinsic to the myotome band itself.

SUMMARY

1. A brief description is given of some features in the special morphological organization of the neuromuscular system in the embryo of the lower vertebrates.

2. The development of locomotory behaviour patterns in elasmobranchs is compared with that in other groups.

3. The earliest movements of the embryo of *Scyliorhinus caniculus* have been photographically recorded; the results of Wintrebert are confirmed. The movements persist unchanged after complete removal of the nervous system; they are rhythmical and independent on the two sides. Any isolated portion of an actively contracting myotome band can act as its own pace-maker.

4. The histological structure of the nerve cord and muscle at the time of these first movements is described in detail. There is strong evidence against any morphological link between the motor neurons and the myotome band; other neurons in the developing spinal reflex arc are similarly unconnected with one another.

5. The results are discussed in relation to similar phenomena in other systems. Consideration is given to the mechanism of the excitation and transmission of the contractile process in the myotome band.

We are greatly indebted for technical assistance in photography and in the construction of the apparatus to Mr G. L. E. Wing and Mr A. Salter.

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A PHYSIOLOGICAL ANALYSIS OF CICADA SONG

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(*Received* 10 *February* 1954)

(With Plate 11)

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INTRODUCTION

According to Myers (1929) the first correct and detailed description of the sound-producing organs of the male cicada was made by Casserius (1600), but credit is usually given to Reaumur (1740), whose account forms the basis for all subsequent accurate descriptions of these organs. Since then a large number of authors (listed by Myers, 1929) have continued the study, and some have attempted to correlate the anatomy with the mechanism of sound-production. There is now general agreement that the spiracular theory of Landois (1867) is incorrect and that, as Reaumur suggested, the sound is produced by the rapid clicking of a pair of drums or tymbals, rib-strengthened chitinous membranes situated on the dorso-lateral surface of what appears to be the first abdominal segment; and that the two tymbal muscles provide the energy for sound-production by pulling on the edges of the tymbals. There are, however, a number of accessory muscles and other structures in the region of the sound-producing organs, and the function of these has been variously interpreted.

Observations on the functioning of the sound-producing organs can be divided into two classes. Felici (1724), Mayer (1877), Lucas (1887) and others experimented by destroying skeletal structures or portions of the musculature and noting the modification of the emitted sound. Myers (1929), Hingston (1922) and others observed living cicadas singing in their natural surroundings and tried to correlate the pattern of the song with movements of the body. Five contributions only in the mass of literature are worth quoting in detail. Distant (1906) provides the standard monograph on the taxonomy; his names and classification are used throughout this paper. Myers (1928) gives the most complete recent account of the anatomy; his terminology is largely adopted here. Carlet (1877) gave an account of the anatomy of several European species and attempted to determine the function of the separate muscles and membranes; his detailed results will be discussed where they are relevant to the present work. Lloyd Morgan (1886) made the important observation that the isolated abdomen of *Platypleura capensis* will 'sing' if the tymbal muscle is irritated or stimulated with a 'weak electric current', but the significance of this result in a preparation devoid of nervous ganglia seems to have been overlooked both by the observer and by subsequent workers. Finally, Pierce (1948), in the course of an investigation with modern recording apparatus of the songs of various insects, measured the physical characteristics of the song of two American species of cicada.

MATERIAL AND METHODS

Species of cicada

The present inquiry was made largely in Ceylon, and is based on an examination of *P. capitata* (Oliv.), which is common in the parks and gardens of Colombo from March to June. This species and the related *P. octoguttata* (Fabr.) were used for laboratory experiments. In addition, magnetic tape recordings, field observations and some anatomical investigations were made on seven other species (Text-fig. 1):

Platypleura westwoodi Stål.
Rihana mixta (Kirby)
Cryptotympana exalbida Dist.
Purana campanula n.sp. (Pringle, 1954*b*)
Terpnosia ransonetti Dist.
T. stipata (Walk.)
T. ridens n.sp. (Pringle, 1954*b*)

Cicadas do not take readily to captivity and survive best when sleeved on to a growing shrub; in cages they soon damage themselves by their own activity. All crucial experiments were therefore made on specimens captured not more than 48 hr. previously.

Electronic apparatus

Stimulators delivering pulses of controllable frequency, amplitude and duration.

Grass Type P-4 pre-amplifier (condenser-coupled) or d.c. cathode follower (Nastuk & Hodgkin, 1950), with the built-in d.c. amplifiers of the Cossor Type 1049 oscilloscope.

M.S.S. Type PMR/1 magnetic tape recorder with Grampion electromagnetic microphone, the high impedance output terminal of the audio-amplifiers being connected direct to the oscilloscope for immediate photographic recording of sounds. These amplifiers have a flat characteristic from 60 to 10,000 cyc./sec. and tests with artificially generated waveforms show that they reproduce pulses at about 5000 cyc./sec. accurately except for the first cycle of the oscillation, which is reduced in amplitude.

Temperature

All experiments were made at the temperature of the laboratory, which was about 30° C.

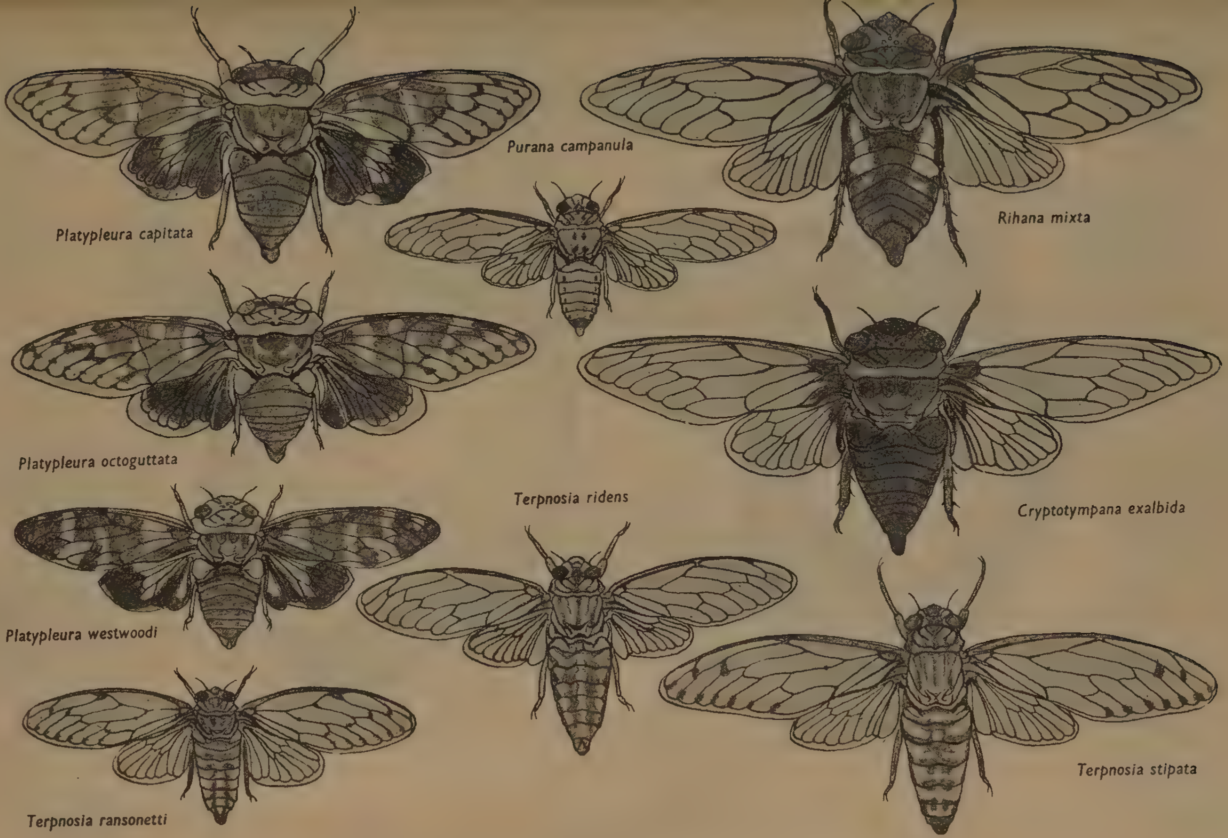
PART I. THE MECHANISM OF SOUND-PRODUCTION

Anatomy and histology

The plan of the organs of sound-production appears to be the same in all species of cicada of which the males sing. It has been described many times; the best general account is perhaps that in Weber (1930). In describing the Ceylon species it will be convenient to list the structures in turn and to indicate their relative positions by reference to figures.

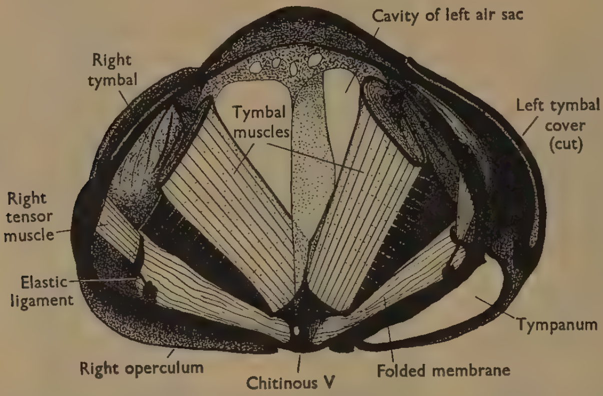
Tymbal (Myers, 1928) (Text-figs. 2, 3 and 5)
syn. 'timbale' (Carlet, 1877), 'schallplatte' (Weber, 1930)

Situated on the dorso-lateral surface of what appears to be the first abdominal segment. Detail of the strengthening ribs varies in different species. The apodeme of the tymbal

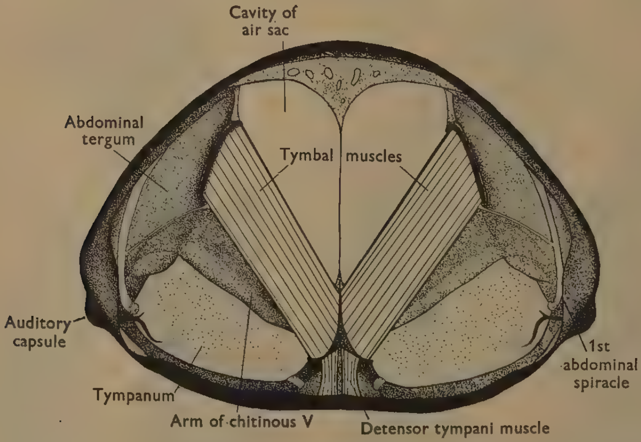


Text-fig. 1. The nine species of cicada studied in Ceylon. ♂♂; Natural size.

muscle inserts at the dorsal corner, from which a strong forked rib runs down to the middle of the posterior edge of the tymbal and acts as a lever, buckling the other, bowed ribs along a line which runs diagonally across the tymbal through two crescent-shaped markings between the main ribs (Text-fig. 5). The change of shape is therefore complex and there may be several modes of vibration.



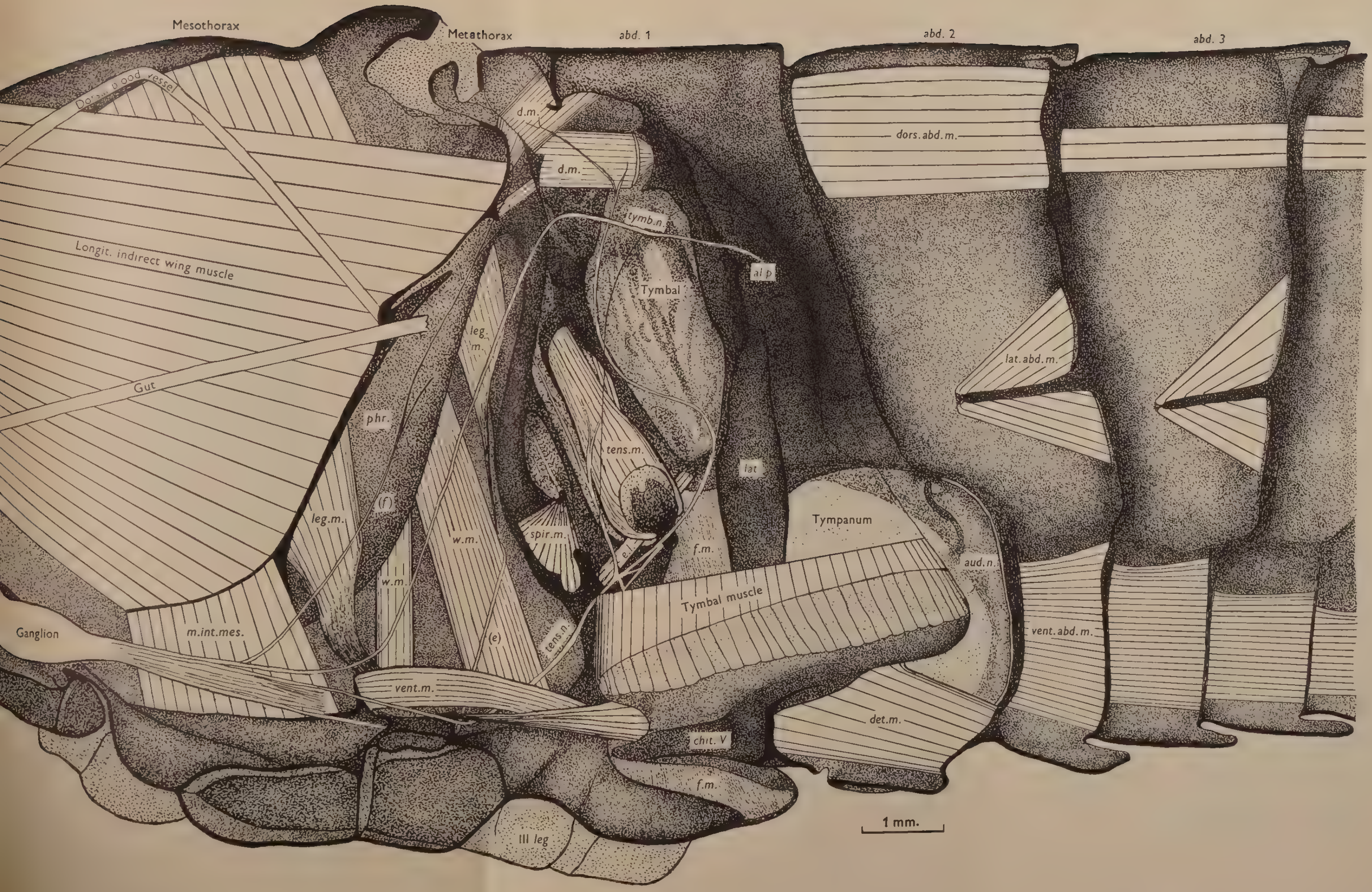
Text-fig. 3. *Platypleura capitata.* Anterior view of a transverse slab including the tymbals and their muscles. The right tymbal cover and the left operculum have been removed, and the dorsal muscles are not drawn.



Text-fig. 4. *Platypleura capitata.* Posterior view of the tymbal muscles, tympana and adjacent structures

Tymbal cover (Myers, 1928) (Text-figs. 1, 3)
syn. 'tympanal covering' (Distant, 1906)

An exoskeletal fold projecting forwards on each side of the abdomen and covering the tymbal. This structure is present only in some cicadas and forms the basis of Distant's

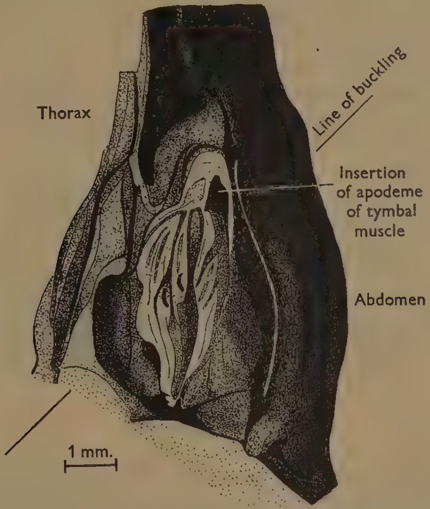


Text-fig. 2. *Platypleura capitata.* Semi-diagrammatic view of muscles and nerves in the region of the organs of sound-production, as seen from within in a specimen bisected longitudinally just to the left of the mid-line. Apart from the thoracic ganglion, gut, blood vessel and the base of the chitinous V, which are shown intact, the remaining structures are duplicated on the left side of the body. The tymbal muscle has been cut across near its base, and the abdominal, auditory and leg nerves are cut near the ganglion. The operculum is not drawn. *abd.* 1, 2, 3, 1st, 2nd, 3rd abdominal segments; *al.p.* attachment point of alary muscle; *chit. V*, base of chitinous V; *e.l.* elastic ligament; *f.m.* folded membrane; *lat.* lateral arm of chitinous V; *phr.* phragma; III *leg*, metathoracic leg. Muscles. *d.m.* dorsal muscles; *det.m.* detensor tympani muscle; *dors. abd. m.* dorsal longitudinal abdominal muscles; *leg.m.* basal leg muscles; *lat.abd.m.* lateral abdominal muscles; *m.int.mes.* 'musculus intersegmentalis mesothoracicus'; *spir.m.* muscle of the metathoracic spiracle; *tens.m.* tensor muscle; *vent.abd.m.* ventral longitudinal abdominal muscles; *vent.m.* ventral longitudinal muscles of the metathorax; *w.m.* metathoracic direct wing muscles. Nerves. *aud.n.* auditory nerve; *tens.n.* tensor nerve; *tymb.n.* tymbal nerve; (*e*), nerve to the direct wing muscles; (*f*) nerve to the anterior dorsal muscles.

(1906) classification, being large in the Cicadinae Dist. (including *Platypleura*, *Rihana*, *Cryptotympana* and *Purana*), small in the Geaninae Dist. (including *Terpnosia*) and absent in the Tibicininae Dist. (including the famous American seventeen-year locust, *Tibicina septemdecim* (L.)).

Operculum (Distant, 1906; Myers, 1928; Weber, 1930) (Text-fig. 3)
syn. 'volet' (Reaumur, 1740), 'opercule' (Carlet, 1877)

An exoskeletal fold of the metathoracic epimeron, projecting backwards on each side ventro-laterally and covering the membranes between thorax and abdomen. The operculum also varies considerably in size in different genera, but its posterior edge is always closely applied to the abdomen, thereby enclosing a space bounded internally by the tympanum and folded membrane. The opercula are rigidly fixed to the thorax and when the abdomen is raised the gap between them and the abdomen is increased.



Text-fig. 5. *Platypleura capitata*. External view of the left tymbal, to show details of the strengthening ribs and the line of buckling.

Chitinous V (Myers, 1928) (Text-figs. 2–4 and 13)
syn. 'furca' (Berlese, 1909), 'entogastre' (Carlet, 1877), 'triangle écailleux' (Reaumur, 1740).

This is a strong skeletal structure forming the basal attachment of the tymbal muscles and projecting dorso-laterally on each side; it is a conspicuous feature of this region of the body and has been variously homologized by different authors. The view taken here, in contrast to all previous workers, is that it is thoracic and is an extreme backwards projection of the metathoracic sternum. This opinion will be elaborated in a later paper (Pringle, 1955) but it is given at once because it affects the view taken of the whole structure of the region.

The arms of the V are firmly attached in the mature adult male to a strong chitinous ridge which forms the posterior edge of the oval in which the tymbal is set, and the arms form a diagonal strut on each side parallel to which runs the tymbal muscle; the muscle is, in fact, folded round the arms of the V, being kidney-shaped in transverse section in its middle region. The strut is the compression member which balances the tension developed in the tymbal muscle.

Ventrally the chitinous V stands on two short processes which reach and join the exoskeleton of the ventral surface; between these processes run the abdominal nerves (Text-fig. 3). The base of the V is in reality double, internal folds of the skeleton having fused in the mid-ventral line giving a strong basal support for the tymbal muscles.

Folded membrane (Myers, 1928) (Text-figs. 2, 3)
syn. 'membrane plissée' (Reaumur, 1740; Carlet, 1877).

A greatly corrugated and opaque membrane separating the air sacs of the thorax from the space enclosed beneath the opercula, and extending on each side from near the mid-ventral line to the lower and anterior edges of the rim of the tymbal. This membrane is stretched when the abdomen is raised.

Tympanum (Myers, 1928) (Text-figs. 2–4)
syn. 'miroir' (Reaumur, 1740; Carlet, 1877).

The membrane of the auditory organ (Vogel, 1923)

Air sacs (Text-figs. 3, 4)

The abdomen of the mature male cicada is filled with large air sacs, which compress the viscera and other organs into a narrow dorsal space. The sacs are closely adpressed to the inner surface of the tymbals, folded membranes and tympana, and surround the tymbal muscles except for a narrow connexion along the arms of the chitinous V. The sacs have usually been regarded as tracheal in nature (Snodgrass, 1921), but Myers (1928, 1929), elaborating a suggestion of Hickernell (1923), regards the sac as mesenteric—an expansion of part of the alimentary canal. This is incorrect, at least in *Platypleura capitata*. The air sacs are paired tracheal enlargements, in communication with the exterior through the metathoracic spiracles.

Tymbal muscle (Text-figs. 2–4)

The main muscles of the sound-producing apparatus, attached basally to the base of the chitinous V and distally to a thin, approximately circular disk from the centre of which a flat apodeme runs to the tymbal. This muscle is fibrillar in structure.

Tensor muscle (Text-figs. 2, 3)
syn. 'muscle tenseur de la membrane plissée' (Carlet, 1877).

A muscle of normal histological appearance running from a well-developed knob on the posterior edge of the metathorax to the anterior rim of the tymbal. Contraction of this muscle has two results. The rim and ribs of the tymbal are further bowed, thereby increasing the tension in the apodeme of the tymbal muscle necessary to click the tymbal, and the whole abdomen is raised, pivoting about its only firm attachment to the thorax in the dorsal region. This movement stretches the folded membrane and increases the space between the ventral surface of the abdomen and the opercula; the mechanism can be seen from Text-fig. 2. The thoracic knob carries the basal insertion of the tensor muscle so far caudad that it lies behind the anterior rim of the tymbal and the muscle slopes forwards and outwards. The chitinous V is carried with the abdomen in this movement, its connexion with the rest of the metathorax being thin and flexible.

Elastic ligament (Text-figs. 2, 3)

A thin, transparent ligament, stretching from the metathorax to the lower rim of the tymbal, which is stretched by contraction of the tensor muscle.

Ventral muscles (Text-fig. 2)

syn. 'muscle sterno-entogastrique' (Carlet, 1877).

Two muscles on each side running from the anterior part of the metathoracic sternum to the base of the chitinous V. These are antagonists of the tensor and dorsal muscles in their action of raising the abdomen; contraction of the ventral muscles holds the abdomen firmly against the opercula.

Detensor tympani muscle (Text-figs. 2, 4)

A short but very stout band of muscle on each side of the mid-ventral line running between the chitinous V and the sternum of the first abdominal segment (the second abdominal segment of other authors). This is the true ventral intersegmental muscle between thorax and abdomen, but since the chitinous V moves with the abdomen it no longer has the function of depressing the abdomen. Instead it acts as a detensor of the tympanum, bending the strong rim which supports this delicate membrane. In the living male cicada it is always contracted before song commences, thereby creasing the tympanum and presumably protecting it from damage by the high intensity of sound.

Dorsal muscles (Text-fig. 2)

These are four in number on each side; two very short muscles lie within the metathorax and two run from the metathorax to the abdomen. The more dorsal of these probably assist in raising the abdomen. The more ventral is a larger muscle whose posterior insertion is on the rim of the tymbal or on a chitinous disk whence a short apodeme runs to the tymbal rim; its contraction increases the curvature of the tymbal and it probably acts synergically with the tensor muscle.

Abdominal muscles (Text-fig. 2)

Between each of the segments of the abdomen run, on each side, dorsal and ventral longitudinal muscles which compress the abdomen and lateral muscles which expand it. The latter are attached to a process which projects forward from the next posterior segment.

Flight muscles (Text-fig. 2) (Weber, 1928; Haupt, 1929)

These comprise, on the one hand, the very large indirect muscles of the mesothorax and the muscle called by Weber the 'musculus intersegmentalis mesothoracicus', which serves to provide a firm anchorage to the base of the large phragma on the anterior surface of which are inserted the fibres of the mesothoracic longitudinal indirect muscle; and, on the other, the direct wing muscles, those of the metathorax only being shown in Text-fig. 2. The metathoracic direct wing muscles serve to unfold and fold the hind wings, but appear to be without significance for the wing-beat (see p. 535).

Nervous system (Text-figs. 2, 6)

The thoracic and abdominal ganglia are fused into a single composite ganglion which lies in the mesothorax and gives off a complex bundle of nerves from its posterior end.

Among these can be recognized:

(*a*) *The main abdominal nerves.* These nerves run as a single trunk through the basal part of the chitinous V and branch only behind the detensor tympani muscle to supply the second and more posterior segments of the abdomen.

(*b*) *The auditory nerve.* This nerve is distinct right from the ganglion but runs with the abdominal nerves through the base of the chitinous V. It diverges in front of the detensor tympani muscles and runs up the posterior edge of the tympanum to the auditory capsule.

(*c*) *The tensor nerve.* This leaves the ganglion near the auditory nerve, runs under the ventral muscles to which it sends a branch, and then turns outwards and upwards to supply the tensor and posterior dorsal muscles. Shortly after leaving the ganglion it gives off a fine branch which joins the tymbal nerve; this is probably the fibre supplying the metathoracic spiracle.

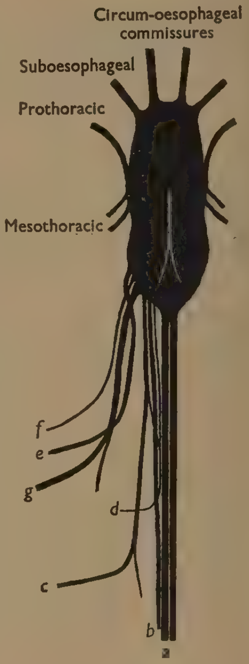
(*d*) *The tymbal nerve.* This large, single nerve fibre has a separate course from the ganglion, emerging near the auditory nerve and running back a short distance before turning dorsad between the air sacs. Reaching the circular disk at the distal end of the tymbal muscle it separates from the fibre supplying the muscle of the metathoracic spiracle and continues round the tymbal muscle to enter the muscle on its posterior aspect. The single tymbal nerve fibre is $15\,\mu$ in diameter; it branches within the tymbal muscle to supply all its fibres.

(*e*) *Nerve to the direct wing muscles.* This emerges from the ganglion more laterally than the nerves already mentioned and runs beneath an internal skeletal process before turning dorsad to the muscles.

(*f*) *Nerve to the anterior dorsal muscles of the metathorax.* A small nerve running up the posterior surface of the phragma.

(*g*) *Metathoracic leg nerve.* This gives off a branch to the basal leg muscles before entering the leg.

The large fibre of the tymbal nerve can be traced forwards in transverse sections of the ganglion right through its most posterior region to the level at which the fibres of the metathoracic leg nerve break up into neuropile; here the two tymbal nerve fibres decussate and then break up into small branches. The fibres of the auditory nerve also form a compact bundle which runs forward to the same region. Binet (1894) described briefly the 'sound-production lobes' in the ganglion and noted a large pair of cells situated laterally at the level of entry of the leg nerve. These may be the cell bodies of the tymbal nerve fibres.

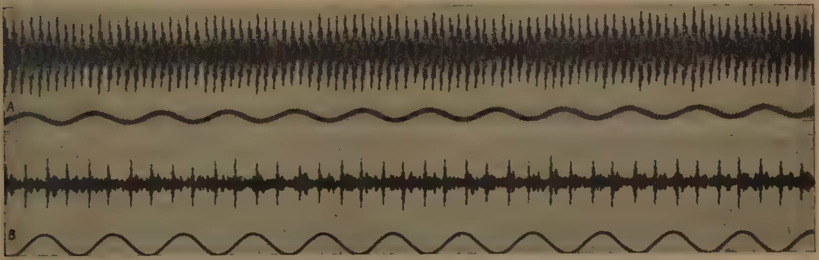


Text-fig. 6. *Platypleura capitata.* Ventral view of the thoracic ganglion showing, on one side, details of the nerves leaving its posterior end. *a*, abdominal nerves; *b*, auditory nerve; *c*, tensor nerve; *d*, tymbal nerve; *e*, nerve to the direct wing muscles; *f*, nerve to the anterior dorsal muscles; *g*, metathoracic leg nerve.

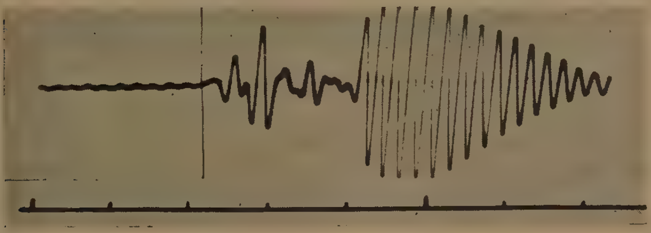
Analysis of the song

The song of cicadas (Pringle, 1953) consists of a succession of short pulses of sound, each composed of a damped train of sound waves (Text-fig. 7). In *P. capitata*, in which the song is continuous, the pulse repetition frequency is about

390/sec. and the sound frequency is about 4500/sec. Field records, in which echoes are liable to affect the waveform of the sound, are not always suitable for high-speed oscillographic analysis, but laboratory experiments with this species show that the amplitude declines approximately exponentially from the start of the pulse (Text-fig. 8). The emission of sound is therefore a free oscillation; its frequency is determined by the natural period of vibration of the tymbal. The sound waveform may be sinusoidal (*Platypleura*) or may show a high proportion of harmonics (*Terpnosia stipata*, Text-fig. 19); in the latter case the tymbal is evidently vibrating in several modes simultaneously.



Text-fig. 7. Oscilloscope records of free song. Time marker 50 cyc./sec. *A*, *Platypleura capitata*; *B*, *Terpnosia ransonetti*.



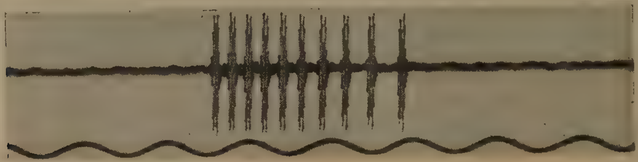
Text-fig. 8. *Platypleura capitata*. The sound emitted during a single IN-OUT click of the tymbal. This recording was made in the laboratory, and is a reconstruction from four high-speed sweeps of the oscilloscope. Time marker 1 and 5 msec.

In captivity or when handled cicadas emit a series of shrieks, which consist of a short train of pulses, often at high frequency. The work of Myers (1929) suggests that in some species there may be several types of song, but in Ceylon this was observed in only one case, *Platypleura octoguttata*, in which the normal pattern of song of the isolated male was replaced by a different rhythm and a lower amplitude of sound when it was sitting on a branch next to a female. The full song of the isolated male, which is the sound usually heard in the field, is characteristic of the species and with practice may be used for recognition. There is surprisingly little variation from one individual to another; the whole pattern of neuromuscular co-ordination is evidently instinctive and inherited with little possibility of modification.

The habits of different species vary. Some, such as *Purana campanula*, sing only in full sunlight, and the forest quietens at once if a cloud shadow passes. Others, such as *Terpnosia stipata*, sing in the evenings, when the males descend from the tree-tops to the trunks of the trees. *Platypleura capitata* sings mainly in sunlight, but a few individuals may be heard late into the evening in Colombo when the population of insects is at its peak.

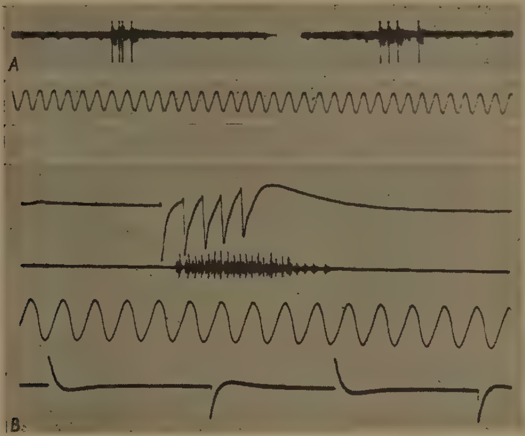
The rhythm of the tymbal muscle

As was suggested by Reaumur (1740) each pulse of sound corresponds to a click of the tymbal. If in a freshly dead cicada the fine apodeme of the tymbal muscle is pulled steadily with forceps, at a certain critical tension the tymbal clicks from its normal resting position (OUT) to a new position (IN) and emits a pulse of sound. As the tension is slowly reduced a second pulse of sound, sometimes at a slightly different frequency, is emitted as the tymbal clicks OUT again. If the tymbal muscle of *P. capitata* is electrically stimulated the double nature of the sound pulses is clearly demonstrated (Text-fig. 8). When this species is thus made to produce sound artificially the amplitude of the IN click is always much lower than that at the OUT movement; other species, such as *P. octoguttata*, have an equal amplitude at IN and OUT. The difference is to be ascribed to the exact form of the buckling which takes place when the tymbal clicks. In the full song of *P. capitata* the pulses become single, for reasons described later, and it is clear that the pulse repetition frequency is that of the rate of contraction and relaxation of the tymbal muscle.



Text-fig. 9. *Platypleura octoguttata*. Rhythmic response of the tymbal muscle to a single stimulus applied to the tymbal nerve. Time marker 50 cyc./sec.

The mechanism of the rhythmic action of this muscle is the first question to be answered in a physiological analysis of the song. In experiments described in full elsewhere (Pringle, 1954*a*) it has been established that the rhythm is myogenic; that is, it is determined by the mechanical conditions of loading and elasticity of the tymbal and not by the synchronous arrival of motor nerve impulses. If the tymbal nerve is cut and single stimuli applied to its distal end, the preparation emits a short train of sound pulses, the frequency declining during the train. As many as ten pulses may be emitted in response to a single stimulus in *P. octoguttata* (Text-fig. 9). Stimulation frequencies of about 120/sec. are sufficient to produce a succession of pulses at a frequency approximating to that of the normal song (*P. capitata*). The rhythmic mechanism is therefore comparable to that of the indirect flight muscles of Diptera (Pringle, 1949) and Hymenoptera (Roeder, 1951). This similarity in physiological mechanism is one of the arguments which leads to the conclusion (Pringle, 1955) that the tymbal muscle is a modified flight muscle and represents the posterior portion of the vertical indirect wing-muscle complex of the metathorax which has become freed from its connexion with flight through the elaboration of a wing-coupling mechanism and has moved caudad at both ends of its attachment, separating the two functions of flight and sound production which were initially combined in a single mechanism. It was established by experiment that a cicada (*P. capitata*) in which all the metathoracic and abdominal nerves had been cut could still fly efficiently, proving that the whole of the power for flight is supplied by the large muscles of the mesothorax, which also move the hind wings through their hook coupling to the front pair.



Text-fig. 10. *Platypleura capitata*. *A*, spontaneous bursts of motor nerve impulses in the tymbal nerve. Time marker 50 cyc./sec. *B*, spontaneous shriek. 1st trace, electromyogram from the tymbal muscle; 2nd trace, sound record; 3rd trace, 50 cyc./sec.; 4th trace, response of amplifier to 5 mV. square waves. The wave-form of the electromyogram is not accurate, due to overloading and short time constant of the amplifier.

During full song, in species in which this is continuous, there is evidently a continuous discharge of impulses down the two nerve fibres to the tymbal muscle. Since cicadas will not sing under laboratory conditions this could not be verified directly, but the descending impulse pattern during a short shriek is shown in Text-fig. 10*A*. This discharge appears as electrical spikes in the tymbal muscle (Text-fig. 10*B*) and is sufficient to put the muscle into rhythmic activity for a short time, producing a shriek. The absence from the electromyogram of potential changes at the frequency of contraction of the muscle is discussed by Pringle

(1954*a*); it indicates that the rhythm derives from properties of the contractile myofibrils and not from a rhythmic depolarization of the muscle cell membranes.

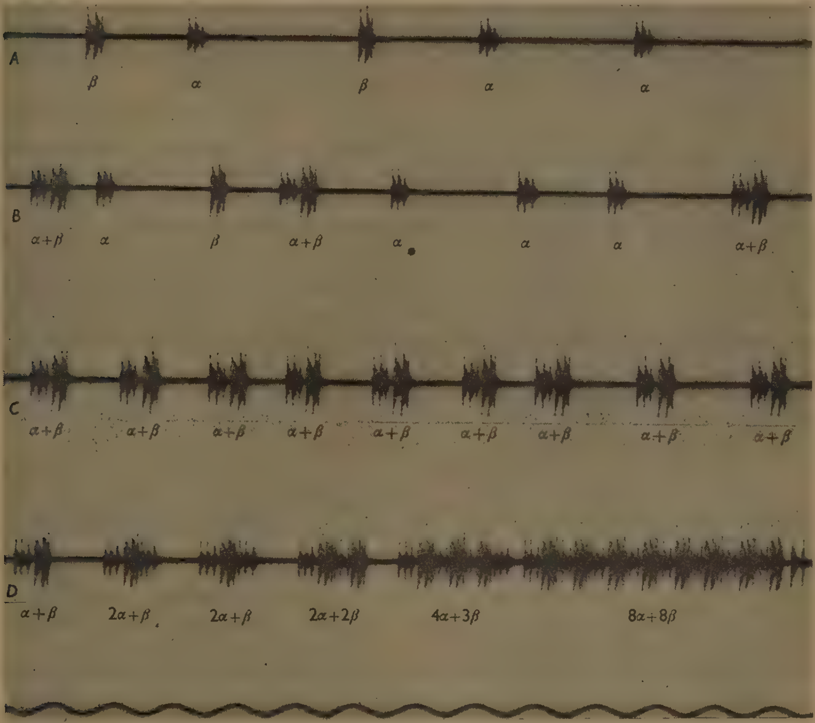
The small impulses in the record of Text-fig. 10*A* are in the motor fibre to the muscle of the metathoracic spiracle, which is maintained tonically closed for long periods.

Synchronization of the two tymbals

Carlet (1877), who had a clear picture of the mechanism of sound production in cicadas, discusses the question of synchronism of action of the two tymbals. He states that in a mature adult cicada the movement of the tymbal during song is too small to be visible, but that in a freshly emerged specimen with incompletely hardened exoskeleton, a considerable distortion takes place. If such a specimen is observed while singing the two tymbals become concave and convex at the same instants. Carlet attempted unsuccessfully to record the tymbal movement on a smoked drum.

Free song records (such as that of Text-fig. 7*B*) show that there is always a single rhythm of sound pulses; the two tymbals must therefore be acting synchronously under normal conditions. This implies both that the two tymbal nerve fibres discharge impulses together and, since the rhythm of the tymbal muscle is myogenic, that some further mechanism ensures synchronism of the actual contractions. Text-fig. 11 shows a case in which the synchronism has broken down. The records were made from a fatigued specimen of *Rihana mixta* held in the hand and stimulated to emit shrieks or single groups of pulses by squeezing the insect; after several minutes of this treatment the two tymbals began to click at different instants and the different amplitudes from the two sides enable the two types of response to be distinguished. They are labelled α and β in Text-fig. 11. Sound is emitted at both the IN and OUT clicks of the tymbal, and a third peak is visible on most of the records, due to some peculiarity of tymbal movement in this specimen; each triple pulse group corresponds to one contraction and relaxation of the tymbal muscle. The records are arranged in inverse order; *D* was recorded earlier in the experiment than *A*. In *A*, *B* and *C* each IN-OUT click of the tymbal represents the arrival of one nerve impulse; this is the usual response of a fatigued preparation (Pringle, 1954*a*). In *D*, before fatigue is so advanced, the later impulses in a grouped discharge evoke more than one contraction of the tymbal muscle. In *A* the tymbal nerve fibres on opposite sides are discharging separately; sometimes in *B* and regularly in *C* the two tymbals click successively with a constant interval of 6·5 msec. There is evidently a tendency for the two nerve fibres to discharge together or nearly together, suggesting the presence in the ganglion of a macrosynaptic connexion between them which is becoming blocked. This functional connexion normally ensures that impulses occur at the same instant in the two fibres, and only when it is fatigued can they discharge separately. Possibly the synapse is situated where the fibres decussate and run close together, as in the case described by Bullock (1953) in the brain of a polychaete, *Protula intestinum*; here two giant fibres have a macrosynaptic connexion which also becomes blocked when the preparation is fatigued.

Synchronism of discharge of impulses in the two tymbal nerve fibres still does not explain the synchronization of muscular contractions in the high-frequency myogenic rhythms associated with full song; this must be due to a mechanical coupling between two autorhythmic systems. The explanation of the myogenic rhythm put forward by Pringle (1954*a*) is that the sudden release of tension in the muscle when the tymbal clicks IN deactivates the myofibrils and leads directly to relaxation. Probably the fact that both muscles are attached basally to a single skeletal structure produces sufficient mechanical coupling between them to ensure synchronization when both are rhythmically active. A similar explanation was proposed by Pringle (1948) of the synchronism between wings and halteres in



Text-fig. 11. Sound records from a fatigued specimen of *Rihana mixta* held in the hand. The pulse groups emitted by the two tymbals are of different amplitude and are labelled α and β. Time marker 50 cyc./sec. *A*, separate contractions of the two tymbal muscles; *B*, where α and β occur together the interval between them is constant; *C*, both tymbal nerves are discharging impulses at the same frequency; *D*, fatigue less developed; repetitive contraction of the tymbal muscles in response to each nerve impulse.

Diptera; here again two autorhythmic systems are mechanically coupled through movements of the exoskeleton.

The function of the tensor muscle

Carlet (1877) called this muscle the 'muscle tenseur de la membrane plissée', and considered that its function was exclusively to stretch the folded membrane and so increase the volume of sound; he detected a slight diminution in sound intensity when the folded membrane was cut. Undoubtedly the tensor muscle has this function since its contraction raises the abdomen, but it also has a direct influence on the mechanical properties of the tymbal.

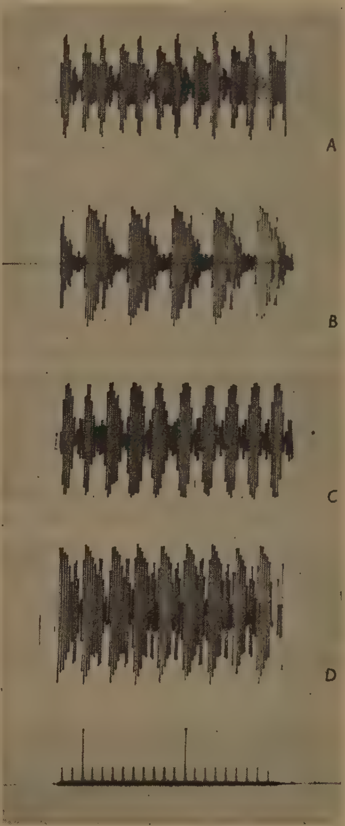
Carlet states that there is no tensor muscle of the tymbal and that such is unnecessary since the concavity of this membrane provides the elasticity needed for its recovery when buckled by the tymbal muscle. Experiments were performed on *Platypleura capitata* to determine whether the tensor muscle increases the curvature of the tymbal and so affects its mechanical properties. The insect was prepared by cutting the nerves just behind the ganglion with a fine pair of scissors inserted through the mesothoracic sternum and removing one operculum to allow electrodes to be placed on one tymbal muscle through the tympanum. Such a dissection upsets the physical properties of the air sacs since one of them is now open to the external air through the tympanum and both through the metathoracic spiracles, whose muscles relax when the nerves are cut. The tymbals and tensor muscles are not, however, disturbed. In the first series of experiments (reported in Pringle, 1954*a*) one tymbal muscle was electrically stimulated and pressure was applied with the point of a needle over the distal attachment of the tensor muscle of the same side, simulating its contraction. These showed a definite decrease in the pulse repetition frequency and an increase in the rate of relaxation of the tymbal muscle after each IN click: results which would be expected if the curvature and therefore the elastic restoring force of the tymbal were increased. In the second series of experiments high frequency (400/sec.) electrical stimuli were applied through a second pair of electrodes to the tensor muscle while the tymbal muscle was strongly excited. The results are shown in Text-fig. 12. At the start of stimulation of the tymbal muscle the pulse repetition frequency was 278/sec. (*A*); when the tensor muscle contracted the frequency dropped to 239/sec. (*B*). As stimulation of the tymbal muscle was contined the frequency rose steadily until it reached 440/sec. (*C*); tensor contraction now reduced this to 408/sec. (*D*). Again it is clear that the tensor muscle, by increasing the curvature of the tymbal and raising the critical tension which must be re-developed in the tymbal muscle before the tymbal clicks IN, has lowered the frequency of the myogenic rhythm.

A further effect is noticeable. When the tymbal muscle of *P. capitata* is stimulated the sound pulses are always double, both the IN and OUT clicks of the tymbal emitting sound, with the IN click smaller in amplitude than the OUT (Text-figs. 8, 12*A*). When the tensor muscle contracts, the amplitude of sound at the IN click increases until it is as large as or larger than that at OUT (Text-fig. 12*B*). The tension required to click the tymbal IN is greater and, when it does click, more energy is dissipated. There is no measurable change in the sound frequency, but the peak amplitude increases about 15%.

These experiments show that the tensor muscle does control the mechanical properties of the tymbal and that its contraction, while the tymbal muscle is rhythmically active, increases the volume and duration of the emitted sound. The volume which could be produced in the laboratory with this preparation was never comparable to that of the full song in the field and other factors must also be involved. One of these is no doubt the resonance of the air sacs (see later), but it may be that contraction of the lower dorsal muscle, which has a tensor action, is also needed to achieve the maximum sound output. This muscle is awkwardly placed for stimulation in an intact insect.

Another function of the tensor muscle, which results from its action in increasing the elastic restoring force of the tymbal, is to balance the tonic tension which is liable to build up in the tymbal muscle when it has been rhythmically active for some time. In the absence of simultaneous tensor contraction this tension is ultimately sufficient to cause a failure of the sound-production mechanism, since, after a time varying from a few seconds to several minutes depending on the state of the preparation, the tymbal may suddenly fail to click OUT again after an IN click; when this occurs the tymbal muscle goes into a tetanus and the rhythmic cycle cannot re-start until stimulation has ceased and re-commenced. The phenomenon was never observed when the tensor muscle was also excited. The physiological implications of the IN failure are discussed by Pringle (1954*a*).

It seems very probable that the tensor muscles are tonically contracted in nearly all species of cicada when they are singing in the field. In some species (see later) analysis of the pattern of song suggests that it is produced by periodic contraction and relaxation of this muscle but, in *Platypleura capitata* and *Terpnosia ransonetti*



Text-fig. 12. *Platypleura capitata.* Sound records from a laboratory experiment illustrating the effect of contraction of the tensor muscle. Time marker 1 and 10 msec. *A* and *C*, tensor muscle relaxed. *B* and *D*, tensor muscle contracted. For further explanation see text.

(Pl. 11, Text-fig. 7, Table 2), where there is no pattern, the apparent singleness of the sound pulses indicates that they are primarily emitted at the IN click of the tymbal, and that the smaller OUT click is lost in the powerful vibration resulting from the IN click.

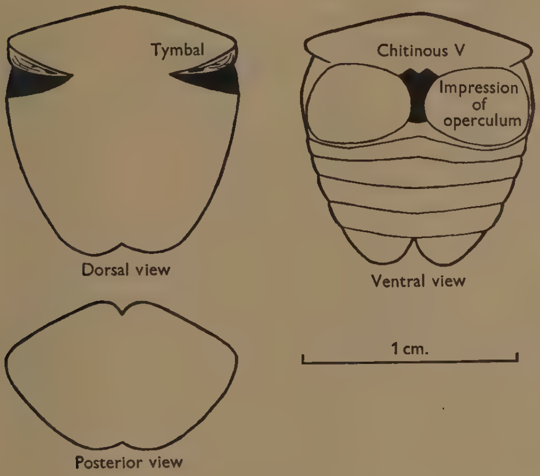
The nature and function of the air sacs

The tracheal nature of the large abdominal air sacs, which was denied by Myers (1928, 1929), has been clearly established. Dissection of fresh specimens showed that they are lined by a membrane which is not wettable by water on the inner (air) surface, but are readily filled with melted paraffin wax when a freshly dead cicada is immersed in this liquid in a flask from which the air has been removed by a suction pump. Experiments with xylol coloured with Sudan III showed that the cavity of the air sac is in communication with the external air through the metathoracic spiracle and that the tymbal muscle derives its tracheal supply from the same spiracle. Injection of coloured xylol into the first abdominal spiracle (lying in the ring of that segment bearing the tympanum (Text-fig. 4)) reddens a tracheal trunk running dorsad to the viscera and sending a branch under the tymbal muscle, but does not colour the fibres of the muscle itself.

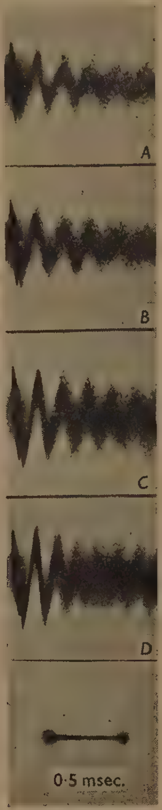
The air sacs must therefore be regarded as of metathoracic origin, their position in the abdomen resulting from their great posterior enlargement. Some light was shed on the manner of their formation by examination of a freshly emerged male cicada in which the walls of the sacs were still folded and partially collapsed. The abdomen was largely occupied by a liquid-filled body cavity, and stretching from the air sacs were numerous very small striated muscle fibres running to the body wall. When they have just emerged, cicadas excrete a large quantity of fluid, which is ejected from the rectum in the form of a fine rain. It is probable that the enlargement of the air sacs takes place partly through the contraction and subsequent absorption of the small muscle fibres, and partly by the excretion of body fluid which draws the walls of the sacs closely into apposition with the body wall, the tymbal muscle and the other internal organs.

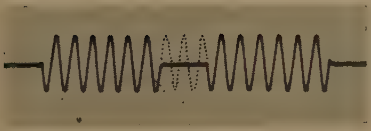
Occasionally specimens were found in which there appeared to be a dorsal median air sac in addition to the two lateral ones. This median cavity has a wettable wall and seems to be the true body cavity into which air has been admitted. Only two out of about 100 specimens examined (*Platypleura capitata*) showed this cavity, and it is possible that its formation was due to an accidental rupture of the delicate lining of the tracheal sacs. Both insects were capable of song.

Paraffin filling of the normal lateral air sacs was used to make a wax cast in order to determine their size and shape (Text-fig. 13). Physically they can be considered as a single cavity, since the membrane dividing them is extremely delicate in the mature insect. This cavity has, on each side at the front end, two openings; a dorsal opening through the tymbal, covered in Cicadinae by the tymbal cover, and a ventral opening through the tympanum covered by the operculum. Experiments were carried out to determine its resonant frequency in a freshly dead cicada, and measurements were also made on a Perspex cavity moulded round the wax cast. The method found most successful was to excite the cavity with a pulsed air jet applied across one tymbal. The sound emitted was picked up with the microphone and observed on the oscilloscope whose time-base was arranged to be triggered by the sound-wave of greatest amplitude. Successive pulses of sound were thus superimposed on the trace and the effect of extraneous noises reduced to a minimum. Specimen records are shown in Text-fig. 14. The frequency of sound emitted in full song by *P. capitata*



Text-fig. 13





Text-fig. 15

Text-fig. 14

Text-fig. 13. *Platypleura capitata.* Outline drawings of a wax cast of the cavity of the air sacs.

Text-fig. 14. *Platypleura capitata.* Determination of the resonant frequency of the air sacs. Superimposed oscillograms of the sound emitted when the cavity is excited with a pulsed air jet. *A*, intact insect; *B*, tymbal covers removed; *C*, tymbals removed; *D*, opercula removed.

Text-fig. 15. Definition of 'coherence'. Diagrammatic representation of two coherent pulses; the dotted section shows the preservation of phase between the two pulses.

varies in different individuals from 4400 to 4700/sec. Measurements of the resonant frequency of the Perspex cavity moulded round a wax cast of the air sacs gave a value of 4220/sec (± 57; four determinations). The values for a freshly dead cicada under various conditions of mutilation are given in Table 1.

Table 1. *Resonant frequencies of the air sacs of a dead cicada* (Platypleura capitata)

Condition	Resonant frequency (cyc./sec.)
Intact insect	5195 ± 29
Tymbal covers removed	5332 ± 52
Tymbals removed	5210 ± 86
Abdomen fully extended	5200 ± 78
Abdomen fully compressed	5560 ± 36
Opercula removed	8290 ± 27
Tympana removed	7562 ± 62

(Standard deviations of the mean; four determinations in each case.)

The method achieves no great accuracy, and the differences between the measurements under the first three conditions are not significant at the 5 % level. Those between the two positions of the abdomen are just significant and the remaining differences clearly so. The biggest effect is produced by removing the opercula, which normally form the lower boundary of the cavity. All the values are higher than those obtained from the Perspex cast of the cavity. Probably the contact between the opercula and the abdomen in a dead specimen is less good than in the living insect (in which the ventral muscles may be tonically contracted) and the resonant frequency is thereby raised; in the cast the cavity is completely sealed at this gap and the frequency is too low. One may conclude that adjustment of the gap in the living insect by means of the accessory musculature provides a means of tuning the cavity to the natural frequency of vibration of the tymbal and so of increasing the volume of the emitted sound. Dilatation of the sacs by expansion of the abdomen appears to have a smaller effect, but might raise the '*Q*' (sharpness of tuning) of the cavity and so reduce the damping.

The function of the tymbal covers is less easy to understand. They may be purely protective. There is no obvious difference in the efficiency of song between cicadas of the genera *Platypleura*, in which they are large, and *Terpnosia*, in which they are small. There is a difference (which may not be general as between Cicadinae and Geaninae) in the quality of the sound and in the manner in which the pattern of the song is generated, but it is not at all obvious how this can be correlated with the presence or absence of tymbal covers.

It is worth mentioning here that air sacs are absent in Fulgoromorph and Jassidomorph Auchenorhyncha, where Ossiannilsson (1949) has shown that the tymbals and tymbal muscles are present, sometimes in both sexes. The song of these small insects consists of a succession of pulses, which are evidently produced, as in cicadas, by the clicking of the tymbals. The damping of the tymbal vibration is, however, high, and no appreciable volume of sound is generated.

Summary of the mechanism of sound-production

(1) The song of cicadas consists of a succession of damped pulses of sound produced by the free vibration of the tymbals as they click either IN or OUT between their resting and a buckled position. The rhythm of tymbal movement is maintained by a myogenic rhythm of contraction and relaxation of the tymbal muscles. The occurrence (but only to a small extent the frequency) of this rhythmic activity is controlled by a single nerve fibre supplying each tymbal muscle; impulses in the two nerve fibres normally occur together, and there is evidence of a macrosynaptic connexion between them in the ganglion.

(2) Of the associated accessory muscles, the tensor muscles (and possibly also a dorsal muscle) by their contraction increase the curvature of the tymbals and so increase the sound output, especially at the IN click. There is an accompanying reduction in the repetition frequency of the sound pulses and a raising of the abdomen in relation to the opercula borne on the thorax.

(3) The air sacs, which are tracheal in nature and open to the exterior through the metathoracic spiracles, form a cavity which is at least approximately resonant to the frequency of tymbal vibration. The resonant frequency is altered by expansion of the abdomen (by contraction of the lateral abdominal muscles) and by raising it, which increases the gap between the abdomen and opercula.

PART II. SONG PATTERNS IN CEYLON SPECIES

The quality of the song to the human ear

Pumphrey (1940) has pointed out that the quality of many insect songs to the human ear may bear little relationship to its significance to another insect. The mammalian cochlea is a frequency-analysing device, each auditory nerve-ending responding maximally to a particular audio-frequency (Galambos & Davis, 1943). The frequency of impulses in a mammalian auditory nerve fibre signals the intensity of sound in a particular range of the auditory spectrum, each ending being coupled to a resonator which is sharply tuned (Gold & Pumphrey, 1948). The ear is thus incapable of registering the frequency of a sound pulse which contains less than a certain number of cycles of vibration.

An air vibration with the waveform of cicada song may produce several sensations to the human listener. Two frequencies are involved; the basic sound frequency (n), which in the species observed in Ceylon lies between 4000 and 7000 cyc./sec., and the pulse repetition frequency (m), which is between 120 and 600 cyc./sec. If the damping of the sound is high (i.e. if the number of cycles of vibration of the tymbal at each click is small) then the human ear registers mainly the pulse repetition frequency. If the damping is low, the most obvious frequency in the heard sound may be that of the tymbal vibration. This is particularly the case if the vibration does not die away completely before the next pulse and if the phase of the sound is preserved from pulse to pulse; such pulses are said to be 'coherent' (Text-fig. 15). If the pulses are coherent then the whole waveform may be considered as a single continuous sound vibration, pulse modulated at pulse repetition frequency.

A Fourier analysis of such a waveform (which is in effect done by the human ear) resolves it into a number of superimposed sinusoidal oscillations; in addition to frequency n there are also present frequencies $n+m$, $n-m$, $n+2m$, $n-2m$, etc., at intensities depending on the exact waveform of the modulation. The human ear registers a musical chord, the quality depending on the harmonics.

If the pulses are not coherent, but nevertheless contain a number of cycles of oscillation, the human ear registers a 'noise', with components at a large number of frequencies including both the sound and modulation frequencies. The sensation is likely to be a sibilant hiss with the much lower modulation frequency superimposed as a faint tone. If there are few cycles of oscillation in each pulse, the main sound heard is the modulation frequency with harmonics of this note.

This digression is necessary in order that the observations reported in the next section may be fully appreciated.

The patterns of song

Magnetic tape recordings of cicadas singing in the field were made for nine species in Ceylon. The distribution and habits of these species are described more fully elsewhere (Pringle, 1954*b*); here we are mainly concerned with the physiological mechanism by which the patterns are generated.

Pl. 11 shows low-speed oscillograms of the song patterns, made from magnetic tape recordings. The significant parameters of the song are summarized in Table 2.

Myers (1929) makes the point that the songs of cicadas are 'no mere mechanical products ground out to a pattern pre-determined by the structure of the sound-organs in the species concerned. The sound-organs themselves are superficially as much alike as a number of violins collected at random; the songs are as diverse as the tunes which may be produced from the instruments by different players.' This view is entirely confirmed by the present study. Small differences in the morphology of the nine species were found, but are not in general sufficient to warrant detailed description. The basic machinery of sound-production consists always of three functional elements:

(*a*) The tymbals and tymbal muscles, directly responsible for the generation of the pulses of sound.

(*b*) The tensor muscles, controlling the pulse repetition frequency and the volume of sound.

(*c*) The muscle complex controlling the resonance of the air sacs.

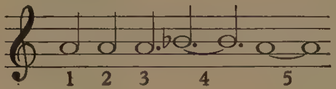
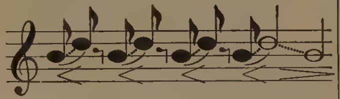
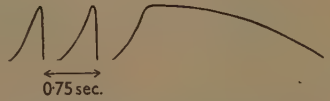
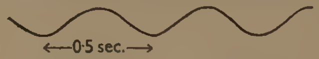
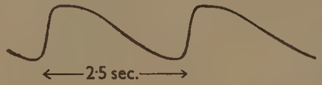
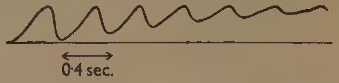
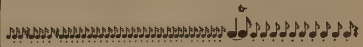
The varied patterns of song are produced by different patterns of excitation of the nerves supplying these three muscle groups.

The species may now be discussed individually.

Purana campanula

This small, slender species (body length 21 mm., width at front of abdomen 6 mm.) is considered first, since its song is the most distinctive to the human ear. The rhythmic unit lasts about 2·5 sec. and comprises a phrase with falling amplitude and pulse repetition frequency (Pl. 11, Table 2). The song has a peculiar bell-like

Table 2. *Analysis of the free song of nine species of cicada from Ceylon*

Species	Sound carrier frequency/sec.	Tymbal muscle contraction frequency/sec.	Pattern of P.R.F. due to action of accessory muscles	Pattern of tymbal muscle excitation
Platypleura capitata	4400–4700	390	Continuous	Continuous
P. octoguttata	5400–5700*	440–480†		Continuous
P. westwoodi	5200	370	Trill at 17/sec.	Continuous
Rihana mixta	4700	295†		
Cryptotympana exalbida	5450	235		Three or four short gaps in excitation at intervals of 0·5 sec.
Purana campanula	4000	280–120		Not analysable
Terpnosia ransonetti	5100	170	Continuous	Continuous
T. stipata	4500–5200	120–300†	Co-ordinated with excitation	
T. ridens	4950	265†	No change in P.R.F.	

* One specimen 7100 (see text).

† Pulses may be double, giving a pulse repetition frequency of twice these values.

quality, the human ear registering not the pulse repetition frequency but the sound frequency modulated at pulse frequency. High-speed oscillographic analysis of the sound (Text-fig. 16*A*, *B*) reveals that the damping is low and that the pulses are coherent. The sound heard is therefore a chord, containing frequencies n (4000 cyc./sec.), $n+m$ (4280 cyc./sec.), $n-m$ (3720 cyc./sec.) and harmonics. The listener naturally pays most attention to the top note of a chord and the sensation is of a note falling in pitch; the simultaneous fall in intensity completes the resemblance to a bell.

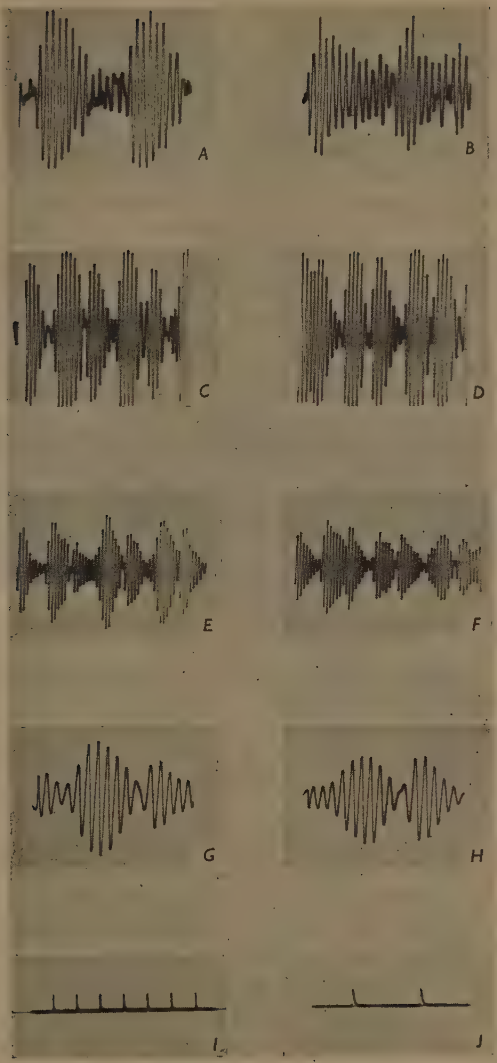
A recently killed specimen of this species was used for experiments on the resonance of the air sacs. The cavity could in this case be excited by plucking the chitin of the abdominal segments with the point of a needle, the sound being recorded on an oscilloscope with triggered time-base. Measurements gave the frequency as 4000 cyc./sec.; the air sacs are therefore closely resonant to the frequency of tymbal vibration. This and the length of the cavity relative to its breadth probably account for the low damping of the pulses.

The coherence of phase between pulses requires explanation, since, according to the explanation of the myogenic rhythm given by Pringle (1954*a*), the IN click of the tymbal occurs at the moment when the tension in the apodeme of the tymbal muscle reaches the critical tension for the click as activation redevelops after the quick-release of the previous cycle; the phase of the free oscillation of the tymbal has no obvious connexion with this cycle. The only satisfactory explanation of coherence is that the oscillation of the tymbal induces a small oscillatory tension in the apodeme which sums with the tension developed by the reactivation of the muscular contractile mechanism. In this way the instant of the IN click will be related to the phase of the previous oscillation, and coherence between pulses is preserved.

Some degree of coherence between pulses probably occurs in some other cicadas in which the damping is sufficiently low for the free oscillation of the tymbal not to have died away completely before the next IN click. But in no other species observed in Ceylon is it sufficiently marked to produce the auditory sensations given by the song of *P. campanula*. It may be noted here that the song of a Japanese species, *Tanna japonensis*, structurally related to *Purana*, has been described (Hearn, 1900, quoted by Myers, 1929) as having a bell-like quality.

Platypleura octoguttata

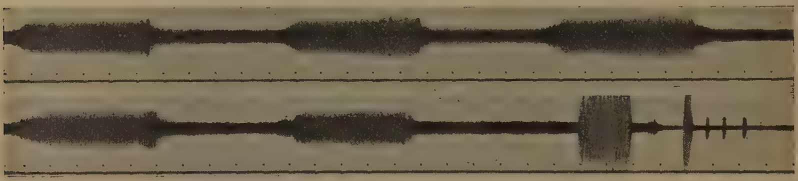
This species provides an extreme contrast to *Purana campanula* in the degree of damping and coherence of the sound pulses (Text-fig. 16, *C*–*H*). In *Platypleura octoguttata* the sound is quenched almost completely between pulses and the most noticeable sound to the human ear is that of the pulse repetition frequency. The song comprises a five-note sequence (Pl. 11) which can best be rendered in musical notation (Table 2). At the fourth note there is a rise in pitch of about a semitone with a drop in amplitude, and sometimes a further increase in the damping. The rhythm of the song is remarkably regular, varying only in the length of the final note (5, Table 2) which may be more prolonged than is shown in the figures.



Text-fig. 16. Coherence of pulses in the free song. *A* and *B*, *Purana campanula*, coherent pulses; *C* and *D*, *Platypleura octoguttata*, pulses not coherent; *E* to *H*, *P. octoguttata* (another insect); *E* and *G*, note 5 of the song (Table 2); *F* and *H*, note 4 of the song. *I*, calibration for *A* to *F*, 1 msec.; *J*, calibration for *G* and *H*, 1 msec.

High-speed oscillograms of the song of this species (Text-fig. 16 *C–H*) provide clear evidence that both the IN and OUT clicks of the tymbal are effective during full song; the pulses are associated in pairs and the waveform resembles that obtained during laboratory experients in which tymbal clicks were induced by electrical stimulation. The frequency of contraction of the tymbal muscle is therefore half the pulse frequency, namely 440–480/sec.

There is no intermission of activity of the tymbal muscle during the song, the apparent breaks in the record of Pl. 11 being due merely to a great reduction in amplitude; the rhythm of pulses is preserved. The abdomen is compressed during the fourth note in which the pulse repetition frequency is raised. Probably the tensor muscle is partially relaxed at this time and the air sacs compressed, reducing the critical tension necessary to click the tymbal and bringing the cavity out of resonance. The overall drop in amplitude of sound bears this out, as does the relative reduction in the amplitude of sound at the IN click (Text-fig. 16 *E–H*). It will be recalled that experiments on the related *P. capitata* (Text-fig. 12) showed that contraction of the tensor muscle increased the energy dissipated at the IN click by increasing the curvature of the tymbal. At the apparent breaks in the song the tensor muscle may be completely relaxed for short periods, so that the clicks are very much less powerful.



Text-fig. 17. *Platypleura octoguttata*. The end of the courtship song; the two portions of the record are contiguous. Time marker 0·5 sec.

P. octoguttata was the only one of the nine species which was observed to sing with a different rhythmic pattern when the male was in close proximity to a female. A small number of these insects were confined on a bush in a garden in Colombo, and on several occasions a male produced what can only be called the 'courtship' song (Text-fig. 17). The volume of sound is considerably less than in the full song and the rhythm is different, consisting of a regular slow alternation of two notes, each lasting about 2·5 sec., one being louder than the other. The courtship song is terminated by a loud shriek, upon which the male attempts to mount the female, which may or may not remain still and allow copulation to take place. The pulse repetition frequency does not change appreciably during the phrases of the courtship song, but rises during the whole performance. It was measured in one specimen as 560/sec. (double pulses) and is therefore higher than in the full song, as would be expected if the tensor muscle is relaxed. The loud and soft alternations are accompanied by expansion and contraction of the abdomen, presumably bringing the air sacs into and out of resonance.

The fundamental sound frequency is higher in *P. octoguttata* than in any other species recorded, in spite of the fact that the insects are large (body length 25 mm., width at front of abdomen 12 mm.). In *P. capitata*, which is little larger (body length 29 mm., width at front of abdomen 12 mm.), the sound frequency is 4400–4700 cyc./sec. The two different specimens of *P. octoguttata* used for the records of Text-fig. 16 gave values of 5700 and 7100 cyc./sec.; other specimens gave 5400 and 5650 cyc./sec. It is possible that the insect giving 7100 cyc./sec. was abnormal in the sclerotization of its tymbal. Resonance of the air sacs was not measured in this species.

Platypleura westwoodi

This is a slightly smaller species than *P. capitata* or *P. octoguttata*, having a body length of 23 mm. and a width at the front of the abdomen of 10 mm. The song was heard on a number of occasions but was recorded only once (Pl. 11, Table 2). Again it seems that the tymbal muscle is continuously active; the pattern is a rapid amplitude modulation at 17/sec. Visual observation during song was not possible, but presumably either the abdominal or the tensor muscles are responsible for the modulation.

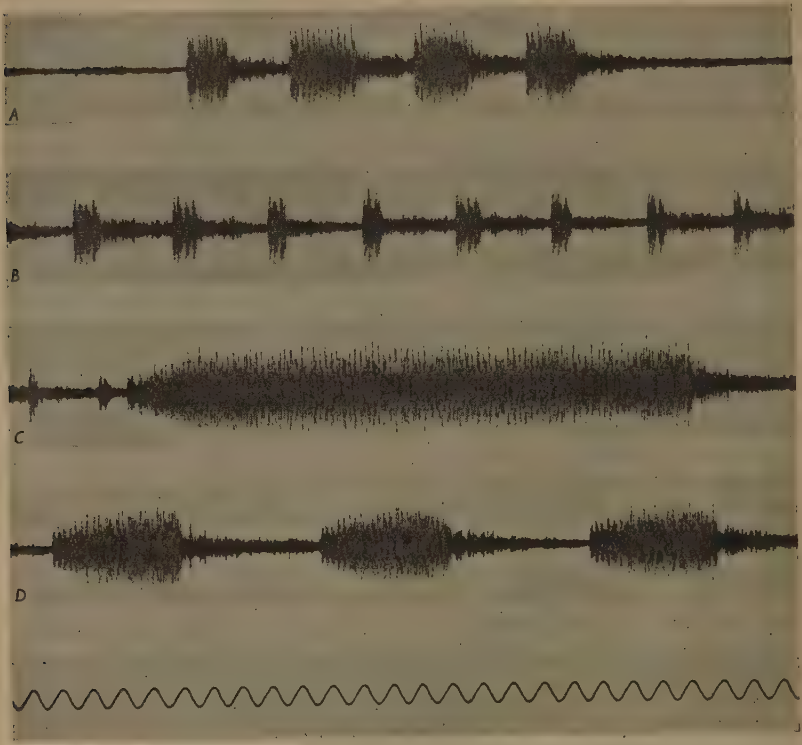
Terpnosia ridens

Terpnosia species have a much narrower abdomen than *Platypleura*; *Terpnosia ridens* measures 27 mm. in body length, but the greatest width of the abdomen is only 8·5 mm. Examination of living specimens of this and other species of the genus suggests that there is less capacity for abdominal dilatation than in *Platypleura* and that the abdomen is almost fully extended throughout life. The song pattern of *Terpnosia ridens* consists of a complex rhythm of pulse groups (Pl. 11, Table 2), with no measurable change in pulse repetition frequency. This suggests that the pattern is one of impulses in the tymbal nerves and that the insect is using the 'on-off switch' to its muscular rhythmic mechanism, rather than, as in *Platypleura*, leaving the switch on and modulating the rhythmic frequency by means of accessory muscles.

The short notes of the pattern (semiquavers, Table 2) contain from two to ten clearly double pulses and may represent single nerve impulses; the long note, in which the repetition frequency is 265/sec. (muscle contraction frequency), has a rising amplitude at the start and a suggestion that the IN click is more powerful than the OUT, so that there may here be a simultaneous contraction of the tensor muscle. Faster oscillograms of different portions of the song are shown in Text-fig. 18.

The basic rhythm of *Terpnosia ridens* contains 47 ± 2 notes of different duration, and is constant throughout the species; no variation outside this range was heard on three occasions on which populations of the insect were observed. The song is produced by each individual for a period of about a minute and is taken up by others, so that a wave of sound passes over a considerable area of forest. If a singing insect is disturbed it flies only a short distance and the song may continue without interruption of the rhythm.

T. ridens, which is a new species, is described in full by Pringle (1954*b*). It differs very little in morphological characters from *T. stipata*, which is more widely distributed in Ceylon and whose song is described next. The morphological differences between these two species, so far as can be determined from the limited material so far available, are so slight that their separation would hardly be justifiable were it not for the differences in distribution and above all in the pattern of song. Since the song is a necessary part of the mating behaviour, the appearance of a new inherited pattern could readily lead to the establishment of a separate interbreeding group, with morphological differences appearing only later.



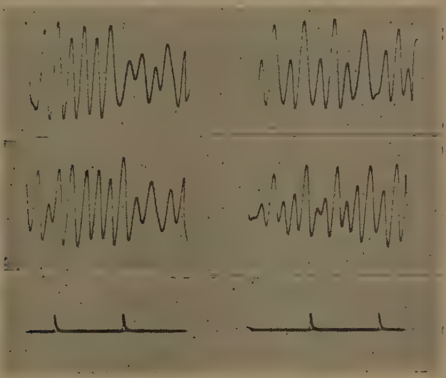
Text-fig. 18. *Terpnosia ridens*. Four sections of the song pattern (see Pl. 11 and Table 2). Time marker 50 cyc./sec. *A*, second short group of semiquavers; *B*, middle of the long group of semiquavers; *C*, long note; *D*, quavers.

Terpnosia stipata

Body length 35 mm.; width at front of abdomen 11 mm.

Again in this species there is evidence of intermittent excitation of the tymbal muscle. The song (Pl. 11, Table 2) consists of the repetition of a phrase lasting 0·4 sec. in which the amplitude and pulse repetition frequency increase. In each period of song the gaps between the phrases become less and less distinct, so that finally the insect is singing continuously with only slight amplitude modulation but with the cycle of pulse repetition frequency still present. Abdominal movement accompanies the song pattern, but is not very marked. The volume of sound is high.

Measurements of the resonant frequency of a dead specimen by flicking the abdominal segments with a needle gave the value of 4400 cyc./sec. The waveform of the sound produced by this species is often far from sinusoidal (Text-fig. 19). In sections where it is sinusoidal the frequency is about 5200 cyc./sec., but elsewhere in the record the fundamental is at about 2250 cyc./sec. with a large component of second harmonic at 4500 cyc./sec. Evidently the tymbal is capable of vibrating in several modes. The strengthening ribs are more heavily sclerotized than in most other species examined, and it is possible that its main mode of vibration is at about 2250 cyc./sec., the second harmonic being boosted by the resonance of the cavity. To the human ear the song has a peculiarly strident quality, which might be expected if the note at 2250 cyc./sec. is accompanied by its octave and other overtones. A similar impression is given by the song of *T. ridens*.



Text-fig. 19. *Terpnosia stipata.* Fast oscillograms showing the sound waveform with high harmonic content. Time marker 1 msec.

The pulse repetition frequency starts at about 120/sec. and rises to 290/sec. in each phrase. Later, when the emission of sound has become practically continuous, the pulses become double, giving a frequency of nearly 600/sec., which is audible to the ear as a high whine accompanying the high frequency noise. This must indicate that both the IN and OUT clicks are contributing to the sound.

Rihana mixta

A large cicada: body length 37 mm., greatest width of abdomen 13 mm.

The morphology of the tensor muscle is different to that of the other species examined; its fibres end basally not directly on the knob of the metathorax, but on a circular attachment disk resembling that of the tymbal muscle, and a short apodeme attaches this to the metathoracic knob. There is no reason to think that this alters the function of the muscle.

The song (Pl. 11, Table 2) consists of two or more short notes, followed by a period of continuous activity, resembling 'hip-hip-hooray' cheering. There is always a period of silence between the notes; this and the quality of the sound, which is not strident, distinguishes it from *Terpnosia stipata*; the duration of the short ('hip') notes is also longer (0·75 sec.). Although to the ear the short notes of the song resemble those of *T. stipata* in that the amplitude and apparent frequency of the pulses increases, high-speed analysis of the sound track reveals a difference. The 'pulses' in this part of the song of *Rihana mixta* are, in fact, groups of pulses (Fig. 20*A*), which merge into continuous activity at the end of the note and during the period of continuous song ('hooray'). Each short ('hip') note must therefore be interpreted as a burst of impulses of increasing frequency in the tymbal nerve fibres, starting at about 30/sec. at which frequency the tymbal muscle gives merely one or a short sequence of contractions at each impulse, and rising to a frequency at which the muscle is maintained continuously active for a short time. There is an accurately co-ordinated elevation and expansion of the abdomen during the short notes—a good example of the way in which the tensor muscle is used to balance the raised mean tension in the tymbal muscle when this is strongly excited.

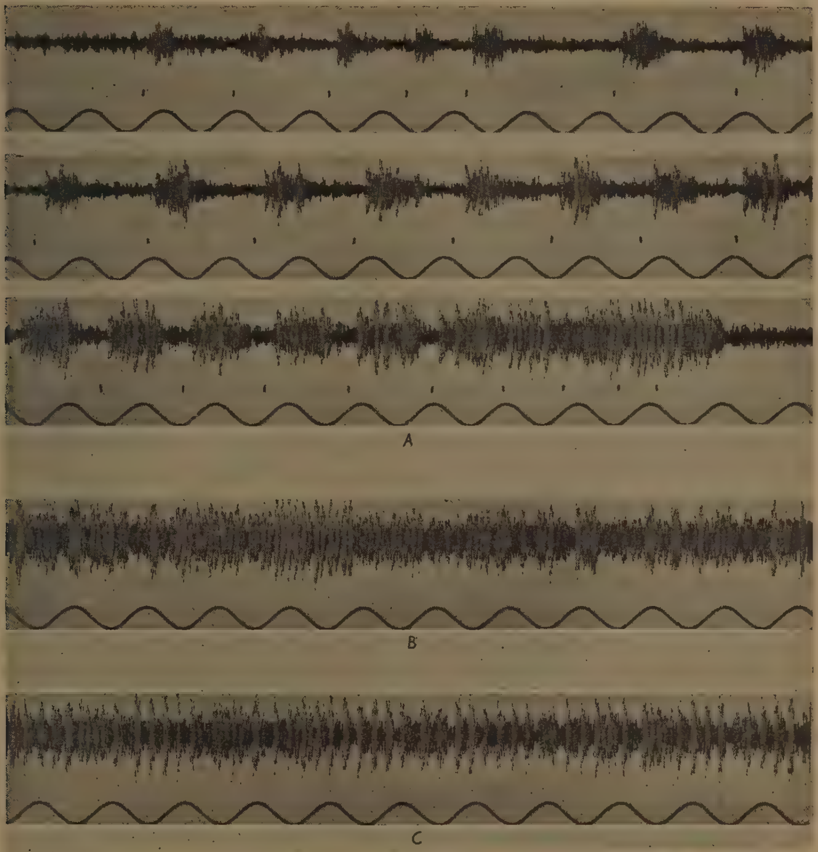
The lack of synchronism between impulses in the tymbal nerves on opposite sides, which was found in a fatigued preparation and is discussed on p. 536, is not detectable in normal song; a single rhythm of pulses always occurs, the maximum frequency of contraction of the tymbal muscle being about 295/sec. The apparent irregularity of Text-fig. 20*B*, *C* arises from the fact that both the IN and OUT clicks of the tymbal emit sound pulses of equal amplitude for part of the time and for the rest one of them is much reduced; the reason for this is not clear, but it is a constant feature of the song of this species.

No measurements were made of the resonance of the air sacs.

Cryptotympana exalbida

Another large cicada; body length 37 mm., greatest width of abdomen 14 mm. The song (Pl. 11, Table 2) has a regular rise and fall in amplitude and pulse repetition frequency every 0·5 sec., accompanied, at the instants of greatest amplitude, by a number of short breaks in the continuity of sound emission. This produces a noise somewhat resembling a knife-grinder. The probable explanation in terms of the neuromuscular mechanism is that the accessory muscles are excited in a 0·5 sec.

rhythm, and the tymbal muscle excitation (frequency of impulses in the tymbal nerve fibres) reduced momentarily to the point where individual nerve impulses evoke a short train of muscular contractions. If this explanation is correct the



Text-fig. 20. *Rihana mixta*. High-speed oscillograms of the free song. Time marker 50 cyc./sec. *A*, consecutive record of a single 'hip' note, showing the composite nature of the 'pulses'. The dots which have been added over the time marker indicate the instants of occurrence of impulses in the tymbal nerves, according to the interpretation given in the text. *B* and *C*, two positions of the 'hooray' note, showing occasional frequency-doubling during periods when both the IN and OUT clicks of the tymbal emit sound pulses.

situation is the inverse of that found in *Rihana mixta*, in which the maxima of excitation of the tymbal and accessory muscles occur at the same instant in the pattern of song; here the minimum of tymbal muscle excitation coincides with the maximum of tensor muscle excitation. The only tape recording which was made is not very suitable for high-speed oscillographic analysis and the estimate of muscle contraction frequency given in Table 2 must be accepted with caution.

Discussion of the song patterns

The nine species recorded in Ceylon reveal a great diversity of song types, and the analysis suggests that the pattern of muscular co-ordination is equally varied. The literature on cicada song from other countries indicates that a similar variety is found elsewhere, but the Ceylon species seem to cover most of the types of song found in the family with the exception of the 'wing-clacking', which Myers (1929) noted in *Melampsalta cingulata*. This is an entirely different method of producing sound which is found only in some cicadas and is probably a true stridulation produced by the rubbing of the wings together or on the sides of the body; a comb-like striated mesonotal ridge which is plucked by a wing-fold has been described in the subfamily Tettigadinae and may represent a development of this accessory method of sound production. Unfortunately the descriptions given by earlier workers without the advantages of oscillographic recording are not sufficiently precise to allow the patterns of song to be analysed in the way attempted in this paper, and no systematic classification of the songs of different subfamilies or genera is possible from a survey of the literature. If recordings become available from a wider range of species, such a classification will be interesting and may help with the taxonomy of the family.

The accurate muscular co-ordination involved in the song patterns indicates a degree of inherited nervous organization which is, at first sight, remarkable, but may in fact be no more complex than is needed to produce particular types of locomotor activity. What is unusual in the animal kingdom is that such a diversity of patterns of co-ordination should have evolved within one family with so little modification of the basic anatomical plan. Fossil cicadas closely resembling present-day forms have been found in Tertiary rocks and doubtful specimens from the Cretaceous (Myers, 1929 review); the family is thus of similar antiquity to the birds, animals which may be compared with cicadas in the way in which complex behaviour patterns have evolved without much change in the structure of the vocal organs.

PART III. SENSORY PHYSIOLOGY

The tympanum of the auditory organ has already been mentioned in the list of structures. It is a delicate membrane situated on each side ventro-laterally under the operculum, and is composed of two layers, the external cuticle and the wall of the air sac, which are sufficiently closely apposed to form interference fringes. An apodeme connects the tympanum to the auditory capsule (Text-fig. 4) containing a large number of chordotonal sensilla, and from these the auditory nerve runs through the base of the chitinous V forward to the ganglion.

Vogel (1923), who made a detailed study of the anatomy and histology of the organ, describes and figures in the male of *Huechys incarnata* A. & S. a tensor tympani muscle running from the dorsal rim of the tympanum to the tergum on each side. This muscle is not present in the mature adult male of *Platypleura* *capitata*, in which also the dorsal rim of the tympanum is formed by the lateral arms of the chitinous V. The muscle is well developed in the female (Pringle, 1955) and was also found in a recently emerged male in which the chitin was incompletely hardened. It seems likely that it is reabsorbed in the mature male of this species after serving to stretch the chitin of the tympanal rim; in *P. capitata* the lateral arms of the chitinous V become firmly attached to the abdominal terga as the chitin hardens (Text-fig. 4), making any tensor muscle unnecessary. Possibly there are specific differences in this point of anatomy.

Vogel misses the function of the detensor tympani muscle, which he calls simply 'längsmuskel', and he does not call attention to the weak point in the tympanal rim near the mid-ventral line (Text-figs. 2, 4) at which movement takes place when the muscle contracts, creasing the tympanum. Otherwise his results were confirmed in the present investigation.

Experimental results

Auditory nerve

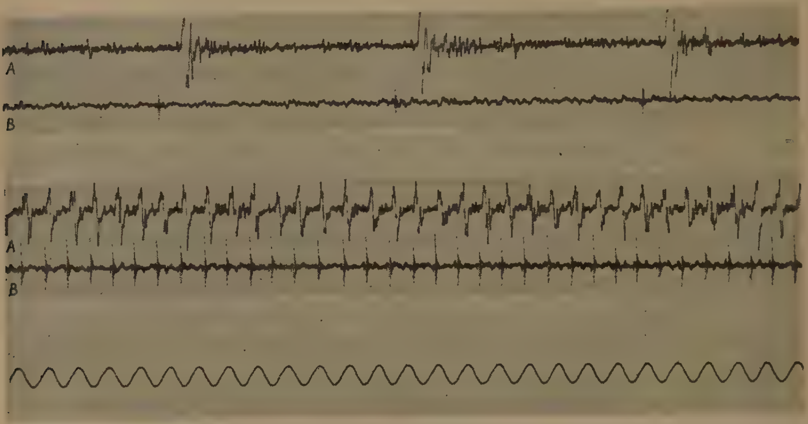
Impulses were led off from the auditory nerve near to its entry into the ganglion. The cicada ear is extremely sensitive to high-pitched sounds, and since no sound-proof room was available it is doubtful if true silence was ever obtained. There was always a background discharge in the nerve, and the impression was gained that any high-pitched sound which was audible to the human ear produced an increase in the nervous discharge.

The discharge obtained in the auditory nerve of a male when the sound of a cicada of the same species was played back through the loudspeaker of the magnetic tape recorder is shown in Text-fig. 21. Each pulse of sound produces a volley of impulses in the auditory nerve, usually with some after-discharge, and the pulses are followed in the nerve up to the highest pulse frequency used (93/sec.). Similar results were also obtained with females. The cicada ear evidently functions, like other insect auditory organs (Pumphrey, 1940), as a rectifier of the received sound, the impulse pattern corresponding to the modulation envelope. The sound frequency in cicada song is therefore that of a carrier wave, and the significant information for the insect is transmitted in the pulse pattern. No attempt was made to determine experimentally whether the auditory receptor of the male is tuned to the sound frequency characteristic of the species, a feature which would increase its sensitivity and selectivity; the fact that the tympana are coupled into the same resonant cavity as the tymbals might increase their response in this frequency range. In the female, where the air sacs are much smaller, this effect could hardly be present unless the tympana themselves contributed to the resonance.

Nerve to the tensor muscles

This nerve, which contains the motor fibres to the tensor and dorsal muscles, has also a larger number of small sensory fibres. In preparations made by bisecting the insect vertically with a razor cut passing just to one side of the mid-line a considerable resting discharge can be recorded from the cut distal end of the nerve.

The endings appear to lie in the region of the skeletal knob on the metathorax to which is attached the tensor muscle. This area is sensitive to vibration, which increases the nervous discharge, but attempts to localize the endings more precisely by mechanical stimulation with the point of a needle were unsuccessful. It is possible that there are near here some mechanical sense organs, of much lower sensitivity than the tympanal chordotonal organs, which act as a monitoring system for the vibrations produced by the insect when it is singing.



Text-fig. 21. *Platypleura capitata.* Electrical record (*A*) from the auditory nerve of a male when the sound emitted by another cicada (*B*) is played back through a loudspeaker. Time marker 50 cyc./sec.

Tymbal nerve

There are no sensory fibres in the tymbal nerves.

Discussion of sensory physiology and behaviour

The results reported in this section are of a preliminary nature only. There is no doubt that both sexes have a well-developed auditory sense; the fact that both males and females of a species are often found assembled into a small group in three or four trees shows that the sense is effective in bringing the sexes together. To what extent the insects react only to the song pattern of their own species is not known, but field observations indicate that the grouping is usually specific. It is noteworthy that where several species of cicada were found in the same locality there was always a clear difference in song pattern between them. *P. capitata* and *Terpnosia ransonetti*, neither of which has any pattern to the song, do not overlap in geographical range. Perhaps the most similar song patterns of overlapping species are *Platypleura octoguttata* and *P. westwoodi*; everywhere where these two species were found together the individuals of the latter were closely grouped in a pure population.

Although the song is almost certainly the stimulus which leads to the assembly of both sexes, it is not established that it provides the females with the only means of locating the males, once they are in the right area. Both chemical and visual stimuli may be involved at the stage of close approach. That females do find the males was made probable by the observation (often made before) that a singing male is frequently accompanied on the branch by one or more females. On two occasions with different species a female was found unexpectedly in the net when a male was captured. It is also clear that the presence of a female does not always lead to a change in the behaviour of the male; the latter may continue to sing at full volume for a long time after females have arrived. Possibly the distinctive courtship song is not present in all species.

The final initiative in copulation comes from the male, at any rate in *P. octoguttata*, in which attempted mounting was observed. For this the stimulus is probably visual except perhaps in those species of cicada in which the female also emits sound by the 'wing-clacking' mechanism.

A point which was not settled satisfactorily is whether the singing male has any facility for monitoring his own song. Although the tympana are creased by the action of the detensor tympani muscles the very considerable vibration in the whole of the skeleton may still be adequate to excite the auditory endings. These or the sensory endings supplied by the tensor nerves could provide a reflex means of adjusting the pattern of co-ordination on the 'reafferenzprincip' (v. Holst & Mittelstaedt, 1950). But it is difficult to see how any of these sense organs could give an indication to the insect of the amplitude of sound energy produced as opposed to the amplitude of vibration at pulse repetition frequency, unless there is such a wide range of thresholds in the auditory chordotonal sensilla that some of them are still within their working range at the intensities of sound produced by the insect itself. It may be that there is a purely instinctive pattern of muscular co-ordination which achieves tuning of the cavity without sensory feed-back; further work is needed to settle this question.

The characteristics of the auditory organ—in particular its reception of merely the modulation envelope of the received sound—imply a lack of sensitivity to the relative phasing of the sound pulses. Coherence or its absence are thus features of the sound which carry no information to another insect. It is also doubtful if the degree of damping of the sound pulses will have much influence on the impulse pattern in the auditory nerve. The inevitable conclusion, that those features of cicada song which have most appeal to the naturalist are those least likely to be important in determining behaviour, may perhaps serve to encourage future workers on these insects to make good use of instrumental aids.

SUMMARY

1. The neuromuscular mechanism of sound-production in cicadas has been elucidated by a detailed anatomical and physiological study of *Platypleura capitata* (Oliv.) and by the analysis of magnetic tape recordings of the song of eight other species in Ceylon.

2. In all cases the song consists of a succession of pulses, the repetition frequency being between 120 and 600/sec. Each pulse is composed of a damped train of sound waves whose frequency is determined by the natural period of vibration of the tymbals.

3. A pulse of sound is emitted when the tymbal suddenly buckles or is restored to its resting position by its natural elasticity; in the song of some species both movements are effective. The tymbal muscles, which are responsible for the buckling, have a myogenic rhythm of activity, initiated, but only slightly controlled in frequency, by impulses in the single nerve fibre supplying each muscle. The two tymbals normally act together.

4. The curvature of the tymbals can be increased by the contraction of accessory muscles, the chief of which are the tensor muscles. This increases the volume of sound emitted at each click and lowers the pulse repetition frequency; the abdomen is raised from the opercula by contraction of the tensor muscles.

5. The tracheal air sacs form a cavity which is approximately resonant to the frequency of tymbal vibration and can be varied in size by expansion of the abdomen.

6. Cicada songs, to the human ear, appear to be of great variety. The differences arise largely from the properties of the mammalian cochlea as a frequency analyser; the degree of coherence of phase between pulses, which is probably without significance to the insect, is of great importance in determining the quality of the sound to a human observer. The songs of three species which resemble respectively a bell, a musical phrase and a strident chatter are analysed from high-speed oscillograms, and the difference in quality of sound is explained by reference to the wave-forms.

7. Some species emit a regular succession of pulses. Others have a slow pattern to their song, produced by the co-ordinated nervous excitation of three functional groups of muscles: (*a*) the tymbal muscles, producing the sound; (*b*) the tensor muscles, controlling the amplitude and pulse repetition frequency; (*c*) the muscles controlling the resonance of the air sacs. Of the nine species recorded in Ceylon, those belonging to the genus *Platypleura* produce their pattern by using (*b*) and (*c*), the tymbal muscle being in continuous rhythmic activity; those of the genus *Terpnosia* use mainly (*a*) to interrupt the continuity of emission of sound pulses, with some accompanying change in amplitude and pulse frequency. The remaining species use all three muscle groups, but different patterns of co-ordination produce great differences in song.

8. In one species (*Platypleura octoguttata*) a distinct courtship song was recorded from a male in close proximity to a female; this ends with attempted copulation.

9. Preliminary electrophysiological experiments show that the chordotonal sensilla associated with the tympana are extremely sensitive to high-pitched sounds. When the song of another cicada is played back through a loudspeaker the impulse pattern in the auditory nerve corresponds to the pulse modulation envelope, with some after-discharge, as in other insect 'ears' (Pumphrey, 1940).

10. The function of the song is to assemble the local population of a cicada species (males and females) into a small group. It remains to be determined whether it is the main intersexual stimulus in mating behaviour.

I am grateful to Prof. Koch and the staff and assistants in the Department of Physiology, University of Ceylon, for the help which they gave me during my stay. The visit was made possible by the award of a Leverhulme Research Fellowship and by grants from the H. E. Durham Fund of King's College, Cambridge, and from the British Council. Part of the apparatus used was purchased with the aid of the Government Grants Committee of the Royal Society. Mrs B. Petrie was responsible for the drawings of cicadas in Text-fig. 1. Dr W. E. China has given me much help in the identification of species.

The magnetic tape recording of cicada songs was played to a meeting of the Society for Experimental Biology in London in January 1954. Gramophone records made from the tape have been deposited with the Colombo Museum. The tape may be borrowed on application to the author at the Department of Zoology, Cambridge.

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EXPLANATION OF PLATE 11

Oscillograms from magnetic tape recordings of Ceylon cicadas. Time marker (for all records) 0·5 sec.

ADDENDUM

(*Received* 12 *October* 1954)

(1) Wakabayashi & Hagiwara (1953) and Hagiwara & Watanabe (1954) have published a study of the physiology of the cicada tymbal muscle in Japanese species. They report that in *Graptopsaltria nigrofuscata*, *Oncotympana maculaticollis*, *Tanna japonensis* and *Meimuna opalifera* twitches accompany each impulse in the motor-nerve fibre, whose natural discharge during 'shrilling' consists of a series of bursts of impulses of about 100/sec. (29° C.). Intracellular electrodes record the normal type of resting and action potential, with little overshoot except in *Platypleura kaempferi*. In a more recent paper (Hagiwara, Uchiyama & Watanabe, unpublished; I am grateful to Dr Hagiwara for showing me the manuscript) *P. kaempferi* is shown to have a myogenic rhythm in the tymbal muscle, and results are described which confirm many of the findings reported here and in Pringle (1954*a*); on the other hand, *Meimuna opalifera* shows always a 1:1 relationship between nerve impulses and sound pulses. If this last observation is confirmed it indicates that the myogenic rhythm is not present in all cicadas, and may necessitate a reinterpretation of the mechanism of song patterning given here for some of the genera other than *Platypleura*.

(2) I have recently been able to examine preserved specimens of two primitive cicadas from Australia, *Tettigarcta crinita* and *T. tomentosa*. The anatomy of the sound-production apparatus in this genus suggests that some of the homologies implied in this paper may need to be reconsidered. The subject will be discussed fully in Pringle (1955).

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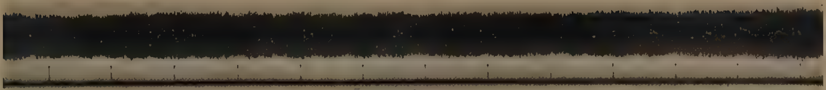
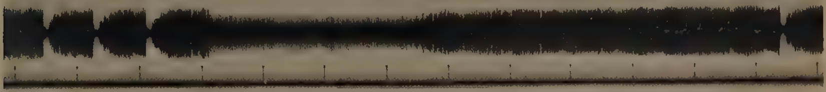
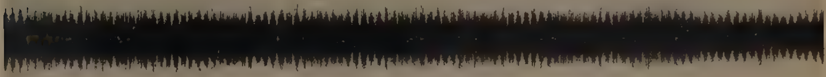


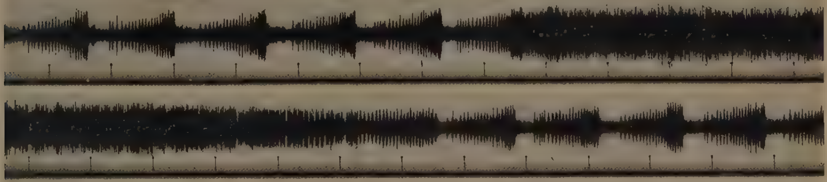
Platypleura capitata



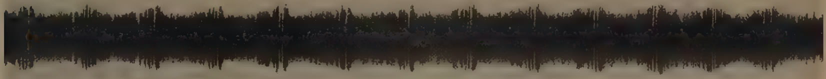
Platypleura octoguttata



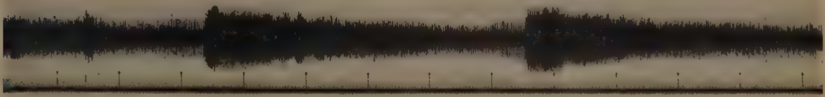
Platypleura westwoodi



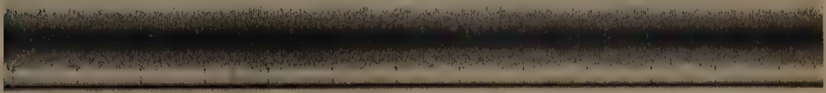
Rihana mixta



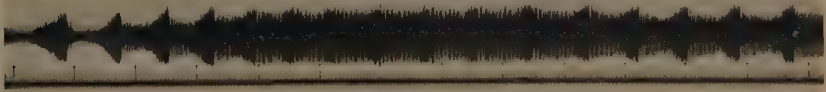
Cryptotympana exalbida



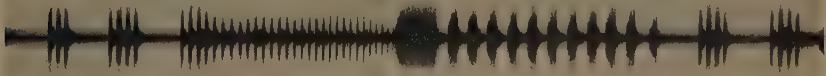
Purana campanula



Terpnosia ransonetti



Terpnosia stipata



Terpnosia ridens

PRINGLE—A PHYSIOLOGICAL ANALYSIS OF CICADA SONG

CONSTITUENTS OF BULL SEMINAL PLASMA

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(*Received* 6 *March* 1954)

INTRODUCTION

Scope. The objects of the experiments described in this paper were to examine: (*a*) the concentration of the inorganic and certain organic constituents in bull seminal plasma; (*b*) the variations in, and correlations between, these constituents in different bulls and the same bull at different times; and (*c*) the freezing-point depression of seminal plasma obtained from different bulls and from the same bull at different times. The results of such experiments are of interest from several points of view. First, semen almost always has to be diluted in experiments on the physiology or metabolism of spermatozoa, and it might be held that the composition of the diluent should bear some relationship to that of the natural sperm medium, at any rate so far as inorganic constituents, substrate and osmotic pressure are concerned. Secondly, semen is always diluted before being used in artificial insemination and similar arguments about the composition of the diluent apply in this case, with certain economic reservations; thirdly, a knowledge of the inorganic constituents of seminal plasma is of obvious importance for future work on the relations between the concentrations of substances within and outside spermatozoa.

Sites of formation. In the bull, most of the seminal plasma originates in the seminal vesicles, the urethral glands and the ampullary glands.

Previous work. Lutwak-Mann & Rowson (1953) made an important contribution to our knowledge of bull seminal plasma by investigating the composition of different fractions of the total ejaculate, obtained separately by electrical stimulation. The first or pre-sperm fraction, originating in the urethral glands, differs greatly in its composition from the whole secretion. These differences are shown in Table 1, together with the results of Mann (1954) and Bernstein (1933) on semen and seminal plasma. Spermatozoa constitute a small percentage, not more than 7% by volume, of bull semen; but if there are particularly high or low concentrations of certain ions within the spermatozoa, the figures for whole semen may be significantly different from those for seminal plasma. At this stage only one comment is needed on Table 1: most of the phosphorus in bull seminal plasma is probably present as phosphoryl choline or glycerylphosphoryl choline (Lundquist, 1946, 1947*a*, *b*; Diament, Kahane & Lévy, 1952), the concentration of inorganic phosphorus being very low, 9 mg./100 ml. semen (Mann, 1954).

MATERIAL AND METHODS

Breeds. Three Shorthorns, four Dexters, one Red Poll, one Friesian and one Ayrshire, making ten bulls in all, were used. Their ages, which varied from 1 year 7 months to 12 years 1 month, are given, with certain other experimental details, in Table 2.

Frequency of collection. Semen was collected once a week, by Mr L. E. A. Rowson, M.R.C.V.S., for 4 consecutive weeks. No other collections were made in the case of the Dexters, but one other was in general made between the experimental collections in the case of the other bulls. The analyses needed about 7 ml. semen per collection and the number of ejaculates was adjusted to meet this requirement. That part of the pre-sperm fraction (Lutwak-Mann & Rowson, 1953) which is secreted before ejaculation was not collected.

Table 1. *Average concentrations of inorganic and certain organic constituents, in mg/*100 *ml., in bull semen, seminal plasma and bovine blood serum*

The figures in brackets refer to the ranges.

Constituent	Semen	Seminal plasma	Pre-sperm fraction	Blood serum****
Sodium	109 (58–201)*	110 (36–230)*	350***	360
Potassium	288 (150–415)*	259 (145–429)*	76·5***	25
Calcium	34 (24–45)*	30 (17–43)*	9·5***	10·5
Magnesium	12**	5*	1·05***	2·2
Chloride	310*	390 (362–433)*	472***	350
Citric acid	720 (340–1150)**	—	—	2·5–5·0
Lactic acid	29 (15–43)**	—	—	11–18
Fructose	540 (280–770)**	—	—	0
Total nitrogen	756**	—	—	46
Total phosphorus	82**	—	—	15
CO_2	16 (ml./100 ml.)**	—	—	50–60 (ml./100 ml.)

* Bernstein, 1933.
** Mann, 1954.
*** Lutwak-Mann & Rowson, 1953.
**** Figures kindly supplied by Dr K. L. Blaxter, the Hannah Dairy Research Institute, Ayr.

Quite apart from linguistic problems, we have experienced difficulties in extracting the figures from Bernstein's paper.

Pre-analysis procedure. After collection, the semen was allowed to cool to 20° C. during about an hour. This unavoidable delay in dealing with the material reduced the concentration of fructose, a point which is considered later; the other constituents under examination were probably not affected by this delay to a significant extent. The semen was centrifuged for 15 min. at 4000 r.p.m. in a refrigerated centrifuge maintained at 19° C., the supernatant seminal plasma being decanted and used in the experiments.

Analytical procedures. Osmotic pressure was estimated by determination of the freezing-point depression, Δ, of three subsamples from each sample. Ramsay's micro-method (1949), which needs less than 0·1 ml. for three determinations, was used. *Potassium, sodium, calcium* and *magnesium* were estimated by the methods described by Robertson & Webb (1939), with minor modifications given by

Rothschild & Barnes (1953), who also give the method used for total phosphorus. *Chloride* was estimated as follows: 0·5 ml. seminal plasma was transferred to a stoppered centrifuge tube and 8 ml. distilled water was added with thorough mixing. The mixture was deproteinized by addition of 2·0 ml. 5 % $ZnSO_4.7H_2O$ solution, followed by 2·0 ml. 0·3 N $Ba(OH)_2$ (Somogyi, 1945). The mixture was left overnight, shaken vigorously at intervals during the first 6 hr., and then centrifuged. 1·0 ml. conc. HNO_3 (freed from oxides of nitrogen by blowing air through it) was added to the supernatant, followed by 5·0 ml. of a standard solution of $AgNO_3$. The precipitated silver chloride was separated by centrifugation and washed 3 times with distilled water, the washings being added to the main

Table 2. *Details of the experiment*

For further explanation, see text.

Bull no.	Breed	Week no.	Age in years and months	No. of ejaculations
1	Dexter	1	12	2
		2		2
		3		3
		4		2
2	Dexter	1	1, 7	4
		2		2
		3		3
		4		4
3	Dexter	1	2, 8	4
		2		3
		3		3
		4		3
4	Dexter	1	8, 1	2
		2		2
		3		2
		4		2
5	Ayrshire	1	3	2
		2		2
		3		2
		4		2
6	Red Poll	1	3, 9	2
		2		2
		3		2
		4		3
7	Shorthorn	1	12, 1	2
		2		3
		3		2
		4		3
8	Friesian	1	2, 2	4
		2		2
		3		4
		4		2
9	Shorthorn	1	2	2
		2		2
		3		4
		4		4
10	Shorthorn	1	2, 8	2
		2		3
		3		4
		4		4

supernatant. The excess $AgNO_3$ was then back-titrated with NH_4SCN, after adding 0·5 ml. of a saturated solution of ferric alum as indicator. The solution was vigorously stirred during the titration. The $AgNO_3$ solution was taken as the primary standard, the NH_4SCN being standardized against it. *Fructose* was determined by the method of Roe (1934) as adapted to semen by Mann (1948). *Citric acid* determinations were carried out by Dr T. R. R. Mann, F.R.S., by the method of Speck, Moulder & Evans (1946). *Iron* was estimated with αα′-dipyridyl. The seminal plasma was wet ashed for iron and the total phosphorus determinations. In the case of the iron determinations, the solution was vigorously boiled to remove excess H_2O_2, after which 1·0 ml. 4% hydroxylamine hydrochloride was added and the solution allowed to stand for 10 min. It was then brought to pH 4·0 with caustic soda, diluted in a standard flask and 1·0 ml. of 1% αα′-dipyridyl in 0·1 N-HCl added. The red colour was allowed to develop for 1 hr. Transmittances were determined with the Spekker absorptiometer, using Ilford green filters, no. 604. Blanks were run and a calibration curve done using $FeSO_4(NH_4)_2SO_4$. Nitrogen was determined by Kjeldahl digestion and distillation in the Parnas-Wagner apparatus, the ammonia being absorbed in saturated boric acid and titrated with N/50-HCl, using a mixed indicator.

With the exception of fructose, the precision of the determinations so far mentioned was ±2%, in the 'non-statistical' sense. The corresponding figure for fructose determinations was about ±15%. This figure refers to different subsamples of seminal plasma, not to different subsamples from the same filtrate. In the latter case the precision would be ±5%.

Sodium and potassium were also estimated with the EEL flame photometer, by Dr J. A. Ramsay, the seminal plasma being diluted 500 times for this purpose and compared with standards containing KCl and NaCl in the proportions which were expected on the basis of Bernstein's data. Chloride was also estimated, by Mr P. C. Croghan, by electrometric ultramicro-titration with $AgNO_3$, an Ag electrode being in the test, and a Pt electrode in the $AgNO_3$, solution (Sanderson, 1952).

Amounts of seminal plasma. The quantities used in the different determinations are shown in Table 3. The advantages of the flame photometer and electrometric micro-titration methods are obvious, so far as amounts of material are concerned.

RESULTS

Freezing-point depression, Δ

Accuracy of the method. Six determinations of Δ were made on a 0·1713 molal solution of NaCl (prepared from the salt dried at 100° C. for 12 hr. before use) in glass-distilled water. Δ was found to be −0·605, −0·60, −0·605, −0·60, −0·60 and −0·60. The value $\Delta = -0{\cdot}60$ is 1·87% higher than that given in International Critical Tables, 1928, for the same solution, and 2·2% higher than that given in Landolt-Börnstein's Tables, 1912. The value agrees, almost exactly, with that obtained by Ramsay (1949). In his paper on this subject, Ramsay says, p. 62, that '...it seems better to treat the new method as a purely empirical one, calibrating the freezing-point depression with solutions of known concentration and accepting whatever relationship gives the greatest consistency'. In view of the close agreement with the other results mentioned above, this attitude seems to be unduly modest.

Δ, *seminal plasma.* The average value of Δ for the forty samples was −0·533, standard error of mean, 0·005.* The range was 0·50–0·71. The value −0·71 only occurred in one sample of semen; without it, the range was 0·50–0·58 and $\bar{\Delta} = -0{\cdot}528$, $s(\bar{\Delta})$, 0·003.

Table 3. *Amounts of seminal plasma used per determination*

Determination	Amount (ml.)	Determination	Amount (ml.)
Δ	0·1 (3)	Chloride	0·5
Fructose	0·1	Chloride (electrometric)	0·1 (3)
Citric acid	0·1	Calcium*	0·75–1·20
Sodium*	0·25–0·40	Magnesium*	1·00–1·60
Sodium Potassium } flame photometer	0·1	Nitrogen	0·2
		Phosphorus	0·2
Potassium*	0·07–0·12		

* Aliquots taken after ashing, etc.
(3), three determinations.

Table 4. *Freezing-point depressions of seminal plasma and semen diluents*

(1) Seminal plasma (these experiments)	−0·53
(2) Sperm Ringer (Mann, 1946)*	−0·53
(3) 3·92 % sodium citrate, pH 6·7 (Burroughs Wellcome and Co.)	−0·69
(4) (3) + equal volume of egg yolk	−0·63
(5) 3·4 % sodium citrate, pH 6·7 (Cambridge and District Cattle Breeders)†	−0·67
(6) (5) + equal volume of egg yolk	−0·64
(7) Phosphate diluent (Phillips & Lardy, 1940)	−0·40
(8) (7) + equal volume of egg yolk	−0·45
(9) SGC-2 dilutor (Milovanov, 1938)	−0·59
(10) Egg yolk (Needham, 1931)	−0·58–0·60
(11) 0·275 M fructose	−0·53

* Without special addition of phosphate buffer or fructose.
† Containing a final concentration of 0·15 % sulphanilamide and 250 i.u. streptomycin calcium chloride/ml.

Δ, *semen diluents.* The freezing-point depressions of several semen diluents were also examined. The results are given in Table 4, from which it is clear that some of the diluents in common use have too high, and others too low, an osmotic pressure. Although bull spermatozoa are said to be rather insensitive to hypertonic media (Lindahl & Kihlström, 1952), the preparation of isotonic media is simple and desirable.

Constituents of seminal plasma

The results of the experiment (standard chemical methods) are given in Table 5, the figures in brackets in column 1 having been extracted from Table 1 for easy reference. Bearing in mind the ranges given in Table 1, and the expected reduction in fructose content referred to on p. 562, the results are in reasonable agreement with those of Mann (1954). This refers to the substances examined by Mann, but not to sodium, potassium and chloride, which were previously estimated by Bernstein. As mentioned earlier, sodium, potassium and chloride were estimated simultaneously by independent methods, sodium and potassium with the flame photometer, and chloride by electrometric titration. The results, given in Table 6, show without doubt that the concentrations of these three substances, given in Table 5, are correct. What has been said above has nothing to do with the goodness or otherwise of the flame photometric and electrometric methods of estimating sodium, potassium and chloride in semen and seminal plasma, nor with the question of their replacing the standard analytical methods. This is discussed later.

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* For the sake of brevity, the standard error of the mean, $\bar{X}$, is referred to as $s(\bar{X})$.

Table 5. *Analyses, bull seminal plasma*

The figures in brackets, column (1), are extracted from Table 1

	Average concentration (mg./100 ml.)	S.E. of (1)	Range of (1)	Average concentration (m.equiv./l.)	No. of determinations
	(1)	(2)	(3)	(4)	(5)
Sodium	258·2 (110)	9·9	152–370	112·3	40
Potassium	171·6 (259)	16·5	50–387	43·9	40
Calcium	37·3 (30)	1·6	24–60	18·6	40
Magnesium	8·4 (5)	0·8	0·1–18	6·9	33
Iron	2·1	0·4	1–4	—	8
Chloride	174·8 (390)	7·7	110–293	49·3	40
Citric acid	620·2 (720)	19·8	357–818	86·6*	40
Fructose	459·7 (540)	29·6	26–872	25·5	40
Total N	876·9 (756)	28·5	441–1169	—	39
Total P	56·9 (82)	2·9	16–91	—	39

* 32·3 mM.

Table 6. *Comparisons of values obtained for sodium, potassium and chloride by chemical analysis (mg./100 ml.), the flame photometer, and electrometric titration*

	Na	*s*(Na)	K	*s*(K)	Cl	*s*(Cl)
Chemical analysis	258	10	172	17	175	8
Flame photometer	267	9	178	17	—	—
Electrometric titration	—	—	—	—	168	8

Correlations between constituents. In spite of seminal plasma being a mixture of the secretions of three separate glands, it is reasonable to ask whether the measurements of any two substances are related, in the sense that when the concentration of substance A varies from sample to sample, the concentration of substance B will also vary, in such a way that the concentration of A has a predictive value with respect to B, or vice versa. The answer to this question is given in Table 7, the most important features of which are: (1) there is a striking inverse relationship between sodium and potassium, which can best be appreciated from the scatter diagram in Fig. 1; (2) there is an interesting relationship between five constituents, which is depicted diagrammetrically in Fig. 2. It would be possible to examine other correlations, e.g. whether any three of the quantities measured are related to

Table 7. *Correlation coefficients between constituents together with their significance levels*

	Na	K	Ca	Mg	Cl	Citric acid	Fructose	N	P
Na	—	−0·86***	—	−0·36*	0·34*	−0·23	—	0·19	—
K	−0·86***	—	0·07	0·42*	−0·24	0·31*	0·14	—	—
Ca	—	0·07	—	0·38*	−0·47***	0·65***	0·61***	0·61***	—
Mg	−0·36*	0·42*	0·38*	—	—	0·42*	0·24	—	−0·07
Cl	0·34*	−0·24	−0·47***	—	—	−0·79***	−0·63***	−0·61***	−0·16
Citric acid	−0·23	0·31*	0·65***	0·42*	−0·79***	—	0·72***	0·57***	−0·09
ructose	—	0·14	0·61***	0·24	−0·63***	0·72***	—	0·60***	—
N	0·19	—	0·61***	—	−0·61***	0·57***	0·60***	—	—
P	—	—	—	−0·07	−0·16	−0·09	—	—	—

*, **, ***, significant at 5, 1 and 0·1 % levels.

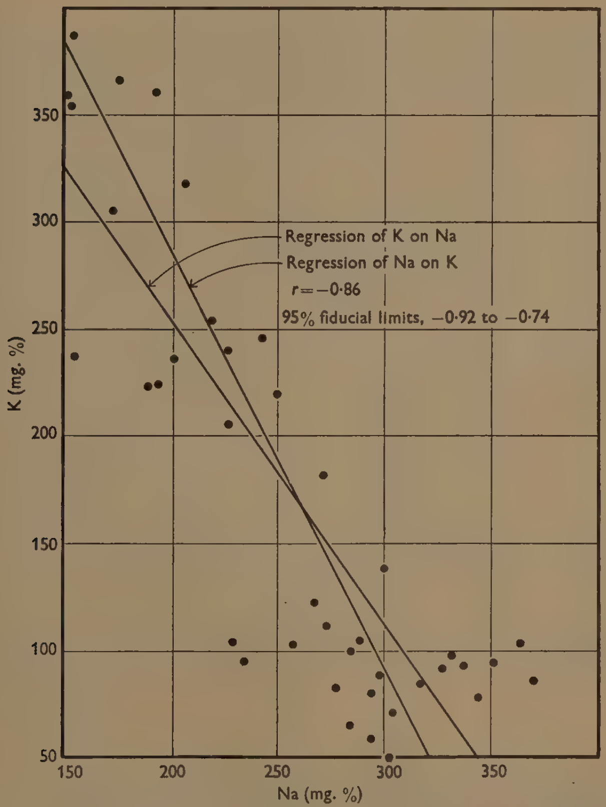

Fig. 1. Inverse relationship between sodium and potassium in forty samples of bull seminal plasma.

each other; but as there are 360 possible analyses of this type and the appropriate sub-routines for a computer are not available, these further examinations have not been made.*

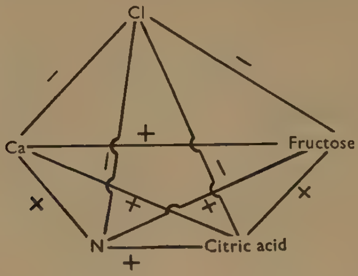

Fig. 2. +, positive correlation; −, negative correlation.

Other correlations. There were no differences between different breeds, or between bulls of different ages; nor trends with time.

Electrometric titration versus chemical analysis. Table 6 shows that there is a 4% difference in the chloride concentration measured by the two methods; the difference is significant. 93% of the variation in the chemical estimations was reflected in the variations in the electrometric estimations. Put in another way, the correlation between the two sets of estimates is high, 0·96. Although it would be of interest to investigate further the cause of the observed negative bias, and of a few 'bad' agreements which occurred, the electrometric method of estimating chloride concentrations, which in its present form needs about 0·03 ml. per determination as against 0·5 ml., can evidently be used with confidence on this material.

Flame photometer versus chemical analysis. The position is less clear in this case. For potassium, the means only differ by 3·5%. There was no evidence of bias and the variation explained was 95%. The correlation was 0·97. Measurement of sodium in seminal plasma with the flame photometer, however, was unreliable, although the means obtained by the two methods happened to be only 3·5% apart. There was no evidence of bias, but the explaining effect of one measurement on the other was only 78% ($r = 0{\cdot}88$). The possibility that the protein content of the seminal plasma might have been responsible for the discrepancies, in spite of the good results with potassium, has been examined. No connexion was found between variations in total N content and cases of bad agreement between the two methods. Until the causes of the sodium difficulty are resolved, or someone else does not have the same difficulties, the flame photometer cannot be recommended for analysing potassium and sodium in seminal plasma, in spite of the attractions of the method.

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* The raw data are available.

DISCUSSION

Δ. Apart from Aschaffenburg's unpublished results using the Hill-Baldes vapour-pressure technique, which are in general agreement with our own (Rothschild & Barnes, 1954), the most recent work on this subject is that of Salisbury, Knodt & Bratton (1948). They measured Δ of bull semen, not seminal plasma, and obtained a mean value of $-0{\cdot}587$. We have found it impossible accurately to measure Δ of bull semen using Ramsay's cryoscopic method, because of the opacity of the semen: in any case, Δ of seminal plasma is more meaningful than Δ of semen, which, in the experiments of Salisbury *et al.* consisted of seminal plasma containing an unknown number of spermatozoa whose effect on the measurements could not be predicted. In their experiments the semen was brought to 0° C. immediately after collection. This treatment must have killed most of the spermatozoa, and it is impossible to say what effect the outward diffusion of intracellular electrolytes and the inward diffusion or adsorption of extracellular electrolytes would have on Δ of the resultant mixture. For this reason, and because Aschaffenburg obtained similar results to ours, we believe that, assuming there is no marked difference between American and British bulls, the value $\Delta = -0{\cdot}53$ is considerably nearer the truth than the value $\Delta = -0{\cdot}59$.

Diluents. As mentioned earlier, Mann's Sperm Ringer is isotonic with seminal plasma, though this is not so in the case of the diluents in commercial use. A mixture consisting of equal volumes of egg yolk and 2·875% (w/v) sodium citrate ($2H_2O$), made up in water containing 0·3 % sulphanilamide, and brought to pH 6·8 with 5·6 % citric acid also made up in water containing 0·3 % sulphanilamide, has $\Delta = 0{\cdot}53$. Other bacteriostatic or bactericidal substances, such as penicillin and streptomycin, are usually added in sufficiently small amounts to make it possible to ignore them from the point of view of Δ. Aschaffenburg (1950) has shown that heating sodium citrate solution does not cause any change in Δ, as claimed by Salisbury *et al.* Heating of the solutions may, however, be an unnecessary refinement.

The analyses show that the most serious defect in commercial citrate buffers is the lack of potassium. As sodium and potassium are present in seminal plasma in the ratio 1·5:1, experiments or trials using sodium and potassium citrate in the appropriate proportions would be worthy of consideration. The experiments of White (1953*a*, *b*) and Blackshaw (1953) support this view. Although the commercial phosphate buffers are entirely unphysiological, they do not affect conception rates adversely (Campbell & Edwards, 1954); but in this case, potassium is present and might counterbalance any adverse effect of the unnaturally high concentration of phosphate.

Electroneutrality. The value 86·6 m. equiv. for citrate in Table 5 is obtained as follows. At 37° C. the dissociation constants of citric acid are: $pK_1 = 3{\cdot}11$, $pK_2 = 4{\cdot}75$, and $pK_3 = 6{\cdot}43$ (Bates & Pinching, 1949). Using the Henderson-Hasselbach buffer equation, one obtains the following values for citrate, in mM./litre at pH 6·7, as the ionic strength tends to zero: $(\text{citrate})^{\equiv}$, 20·9; $\text{H}(\text{citrate})^{=}$, 11·2; $\text{H}_2(\text{citrate})^{-}$, 0·3.

In view of the ranges and standard errors given in Table 5, the citrate figures are not worth correcting for the ionic strength of seminal plasma. The total anions therefore amount to 136 m.equiv., and cations to 182, an anion deficit of 46. This fairly small deficit is presumably made up by a small phosphate, lactate, amino-acid and bicarbonate contribution, and by proteins.

Osmotic pressure. The freezing-point depression of a fully ionized solution of NaCl and KCl containing the amounts of Na and K found in seminal plasma would be about $-0{\cdot}55$. Bearing in mind that this calculation takes no account of the contribution to the osmotic pressure of calcium, magnesium and fructose, it seems reasonably certain that a significant amount of the sodium and/or potassium present is in unionized form. One way of further investigating this point would be by determination of Δ for the appropriate mixtures of sodium and potassium citrate.

'Control' of seminal constituents. When one remembers that seminal plasma is derived from at least three different glands, it is difficult to understand the intimate inverse relationship between sodium and potassium, let alone that between calcium, chloride, fructose, citrate and total nitrogen. Superficially, it might seem that the inverse relationship between sodium and potassium is determined by the proportion of pre-sperm fraction in seminal plasma, which probably varies in spite of the precautions which were taken to avoid collecting it. As the concentration of sodium is 350 mg./100 ml. in the pre-sperm fraction, a high proportion of pre-sperm fraction will cause a high sodium concentration and a low potassium concentration in the seminal plasma. The low potassium concentration will be caused by *dilution* of the K present in the secretions of the seminal vesicles and ampullary glands. (The pre-sperm fraction must dilute the potassium present in these latter secretions, as the final product has 2·2 times as much potassium as there is in the pre-sperm fraction.) This argument, about the pre-sperm fraction controlling the inverse relationship between sodium and potassium, breaks down when consideration is given to the chloride situation. If the argument were correct, a high concentration of sodium in seminal plasma would be correlated with a high concentration of chloride (because there is 2·7 times as much chloride in the pre-sperm fraction as in seminal plasma) and chloride in seminal plasma would be inversely correlated with potassium. Neither of these relationships occurs and some more subtle explanation of the sodium-potassium correlation must therefore be sought.

The position is just as obscure in the case of the other five constituents whose concentrations are locked together. With the exception of fructose and citrate, which are both secreted by the seminal vesicles and 'controlled' by testosterone (Mann, 1948), or possibly of citrate and calcium, there is no *a priori* reason to expect, for example, that a knowledge of chloride concentration should enable one to predict the average concentrations of calcium, total nitrogen, citrate and fructose for the bull in question. One must conclude that the various accessory glands cannot be considered independently, and that some form of over-riding 'control' is exercised on these secretions.

SUMMARY

1. The concentrations of the main inorganic and certain organic constituents of bull seminal plasma have been examined. The average concentrations, in mg./100 ml., were:

Sodium	258	Chloride	175
Potassium	172	Citrate	620
Calcium	37	Fructose	460
Magnesium	8	Total N	877
Iron	2	Total P	57

2. Sodium and potassium were found to be inversely correlated, $r = -0{\cdot}86$, $p < 0{\cdot}001$.

3. Calcium, chloride, fructose, citrate and total nitrogen were also strongly correlated ($p < 0{\cdot}001$), in such a way that a knowledge of the concentration of any one of the constituents enabled the average concentration of the other four in the sample to be predicted with considerable accuracy.

4. Sodium, potassium and chloride were estimated by standard chemical methods and with the flame photometer (sodium and potassium) and by electrometric titration (chloride). The applicability of the alternative methods of estimation, which require very small quantities of seminal plasma, has been examined and is discussed.

5. The freezing-point depression, Δ, of bull seminal plasma was $-0{\cdot}533$, standard error of mean, 0·005.

6. The Δ's of a number of laboratory and field diluents were also examined; with two exceptions they were found to vary markedly from Δ for bull seminal plasma.

We are indebted to Mr J. S. Chivers and Dr A. Walton for allowing certain of their bulls to be used in these experiments; to Mr P. C. Croghan for carrying out the electrometric estimations of chloride; to Dr T. R. R. Mann, F.R.S., for the citrate analyses; to Dr J. A. Ramsay for the estimations of potassium and sodium with the flame photometer; to Mr L. E. A. Rowson, M.R.C.V.S., and Mr D. L. Milne, of the Cambridge and District Cattle Breeders Ltd., for collecting the semen; and to the Medical Research Council for financial support.

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THE STRUCTURE OF THE YOLK OF THE HEN'S EGG, INVESTIGATED BY MEANS OF ITS PERMEABILITY TO ^{82}Br

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(*Received* 23 *June* 1953)

INTRODUCTION

The white and yolk of the freshly laid hen's egg are in a state of physico-chemical disequilibrium with one another. Not only is there a difference in the freezing-point of the two media, $-0{\cdot}46$ and $-0{\cdot}56°$ C. respectively, and in their pH, 9 and 6, but inequalities are found in the concentrations of all the readily diffusible ions that have been analysed (Needham, 1931 *a*). These differences are not abolished in the infertile egg after months of storage, and the reason for this has given rise to much speculation; this has been discussed at length by Needham & Smith (1931) and more briefly by Needham (1931 *b*, 1942).

An early hypothesis, that the maintenance of the disequilibrium resulted from the activity of the membrane enclosing the yolk, the vitelline membrane, fell into disrepute when no signs of such a metabolic activity could be detected. In its place it was assumed that some physical property was responsible, almost certainly a barrier to diffusion. It was not agreed, however, where this barrier was located; Hardy (1933) and Capraro & Fornaroli (1939) believed it to be in the mass of the yolk while others, for example Orrù (1939) and Klissiunis (1948), in the vitelline membrane. Needham (1931 *b*) found that the isolated vitelline membrane permitted rapid equilibration of salt solutions placed on either side of it, and that when they were replaced by white and yolk, equilibrium was reached more slowly though more rapidly than in the intact egg. He concluded that both components had some physical structure, not wholly destroyed when they were removed from the egg, which was responsible, in co-operation with the membrane, for the delay in equilibration.

Recently, one of us (Maurice, 1952) showed that the electrical resistance of the intact yolk was extremely high while that of the membrane was negligible, and suggested that the barrier to diffusion lay wholly in the former. The experiments described below were designed to test this hypothesis directly by measuring the rate of penetration into the yolk, and its distribution after penetration, of a diffusible substance introduced into the white. To ease the problem of chemical analysis it was decided to use a radioactive test substance, and ^{82}Br as the bromide ion was chosen on account of its chemical inertness, high radioactivity, and reasonably long half-life (34 hr.). It was found, on analysis of the results, that because of the slowness with which diffusion takes place into the yolk it would have been valuable to have a longer lived isotope; nevertheless, it was confirmed that the resistance to diffusion lay in the yolk itself, and further information about the nature of this resistance was obtained.

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This work has already been briefly described in Italian and English (Maurice & Fidanza, 1952*a*, *b*).

METHODS

A solution of $NH_4{}^{82}Br$ was made up isotonic with egg white, adjusted to pH 8, and coloured with Evan's blue. At first water was used as the solvent, but in order to raise the specific gravity of the solution and thus promote readier mixing with the white, dialysed egg albumin was preferred in the later experiments.

To inject an egg the shell was opened at one end and 5 ml. of the white taken up into a syringe containing $\frac{1}{2}$ ml. of the test solution. The contents of the syringe were shaken together until uniformly coloured. The mixture was then re-injected through a long curved needle as equally as possible throughout the white remaining in the shell. Further 5 ml. samples were then withdrawn and re-injected at random until no more large uncoloured volumes could be seen.

To separate the yolk for analysis at the end of the experimental time it could have been either rinsed free of white or else broken free after the whole egg had been boiled or frozen hard. Some yolks were rinsed with saline in preliminary tests, but the method was rejected because of the difficulty of adjusting the conditions so that all the adherent white was removed while none of the ^{82}Br was washed out of the yolk. Boiling was excluded from practical consideration because a marked and indeterminate diffusion could occur while the egg was being heated. There remained the possibility of freezing the egg solid and this was found satisfactory in practice. The egg was dropped into a mixture of 'dry ice' and ether, and solidified to its centre in a few minutes. With a sharp blow the yolk could generally be detached cleanly from the white, the vitelline membrane invariably remaining attached to the latter. The frozen yolk was wiped free of any particles of white adhering to it, melted in a measuring cylinder and homogenized with, as a rule, 4 times its volume of 1 % saline. The white was diluted some hundreds of times in water to give a solution similar in radioactivity to that of the yolk. These activities were determined by means of a cylindrical Geiger–Muller counter dipping into the liquids; no particular refinements of technique were adopted since a standard error of 10 % in the measurement of the ratio of the ^{82}Br concentrations in the yolk and white was considered permissible in view of the much greater variations found between eggs.

Throughout the experimental time, for a period of temperature equilibration beforehand, and during the separation of the yolk from the white, the eggs were in a cold room maintained at a temperature of 1° C.

All the eggs were obtained from the same stock of Italian Leghorns during the period March to April. With a few exceptions to be noted later they were injected on the day they were laid.

RESULTS

Theoretical

The principal object of these experiments was to determine whether the greater part of the resistance to the diffusion of ^{82}Br into the yolk is distributed throughout its mass or is located in a layer surrounding it, the vitelline membrane. If the average concentration of the ion in the yolk, C_y, remains small in comparison with the value it would attain when in equilibrium with the white, it follows from the simple laws of diffusion (Jacobs, 1935) that when the resistance is entirely in an external membrane of permeability K:

$$C_y = \frac{A}{V} K t C_w. \tag{1}$$

When it is entirely in the mass of the yolk where the diffusion constant for the ions is D:

$$C_y = \frac{2A}{V} r \left(\frac{Dt}{\pi}\right)^{\frac{1}{2}} C_w, \tag{2}$$

which is equivalent to assuming that the yolk is an infinitely thick layer and that its interface with the white is plane. In these equations V and A represent the volume and superficial area of the yolk, r the ratio of the concentration of ^{82}Br in the yolk to that in the white at equilibrium, t the experimental time, and C_w the average concentration in the stratum of white immediately in contact with the yolk; this must stay constant throughout the experiment, but need not be equal over the surface of the yolk so long as the resistance to penetration is everywhere the same.

These equations may be rewritten:

$$\log \frac{C_y}{C_w} = \log t + \log \frac{A}{V} K, \tag{1a}$$

$$\log \frac{C_y}{C_w} = \tfrac{1}{2} \log t + \log \frac{2A}{V} r \left(\frac{D}{\pi}\right)^{\frac{1}{2}}. \tag{2a}$$

If the concentration ratio is plotted against time on logarithmic paper the equations represent two straight lines one having twice the slope of the other. To which of these lines the experimental values correspond most closely should indicate, therefore, which of the two hypotheses should be preferred.

It may be noted that if both types of resistance to diffusion are present together, for example, a resistive membrane surrounding a mass of yolk which is itself an appreciable obstacle to diffusion the experimental points should first follow a line of higher slope corresponding to the membrane, then, as the resistance of the yolk becomes relatively more important, one of the lower slope.

Experimental time

Since the experimentally determined concentration ratios showed a wide scatter it was desirable to measure their variation with time over as wide a range as possible. The decay in radioactivity of the ^{82}Br imposed an upper limit on the experimental time of 200 hr. after which the activity of the yolks became too weak to assay. The lower limit is a result of the concentration in the stratum of white in contact with the yolk not immediately acquiring a constant value as required by equations (1 *a*) and (2 *a*). Thus, in eggs that had been frozen shortly after their injection, it could be seen from the areas coloured with Evan's blue that the injected material was distributed fairly uniformly throughout the white but that it failed to mix with the layer of thicker albumen which directly covers the yolk.

To determine how quickly a constant value for the ^{82}Br concentration was attained, the inner surface of the frozen white of some of these eggs was scraped off as uniformly as possible with a scalpel until about 0·2 ml. had been collected. With a pipette, 0·1 ml. of the melted scrapings were separated, diluted, and analysed separately from the mass of the white. In eggs frozen 5, 2, and 1 hr. after injection (three in each case), the concentration of ^{82}Br found on the inner surface was equal to that in the rest of the white. In two eggs frozen after 10 min. the inside concentrations were 60 and 68 % of the rest, and in one after 5 min., 56 %. It seems likely, then, that the constant value, 100 %, would be approached shortly after 10 min., and the shortest experimental time for which the equations could be considered valid was taken as 1 hr.

It is possible that the permeability of the yolk changes after the egg has been laid. Then the results obtained after long and short experimental times would not be comparable. To test this, a few eggs which had been kept in the cold room for 200 hr. were injected with ^{82}Br and frozen after a short time. After 2 hr. the average concentration in the yolk of three such eggs was $1\frac{1}{2}$ times greater than that found in five freshly laid eggs, largely as the result of one very high value; after 24 hr. the average of three older yolks was only 10 % above that of four fresh ones. These greater penetrations are not very significant in comparison with the scatter of the individual values and it was assumed that any ageing that occurred could be ignored.

Experimental values

The values found experimentally for the ratio C_y/C_w from 1 to 200 hr. are shown as the points in Fig. 1. A straight regression line was fitted to all these points and had a slope of 0·75; it is drawn as the continuous heavy line. This slope is midway between those predicted by equations (1 *a*) and (2 *a*), 1 and 0·5, and it is statistically extremely improbable that it represents a random variation from either of them. It is evident, therefore, that a mechanism more complicated than either of those considered above, acting singly, regulates the penetration of ^{82}Br into the yolk over the whole of this period. As the points do not lie equally about the straight line fitted to them but conform to a curved line, there is a possibility that one of the two types of resistance could be controlling the penetration over a limited range. Accordingly another regression line was fitted to those points corresponding to the fifth hour onwards and had a slope of 0·91; it is drawn heavy and broken in the figure. This is not an improbable variation from the slope of 1·0, being just not significant at the 5% level, and the agreement would be better if it is allowed that the disturbing influences operated in a lesser degree after the fifth hour. Over this limited time, then, the passage of ^{82}Br into the yolk was at a constant rate as if it were across a resistive membrane; from the position of the regression line and the average volume of the yolks (18 ml.), the permeability of this membrane to the ion, K, was calculated to be 7×10^{-5} cm./hr.

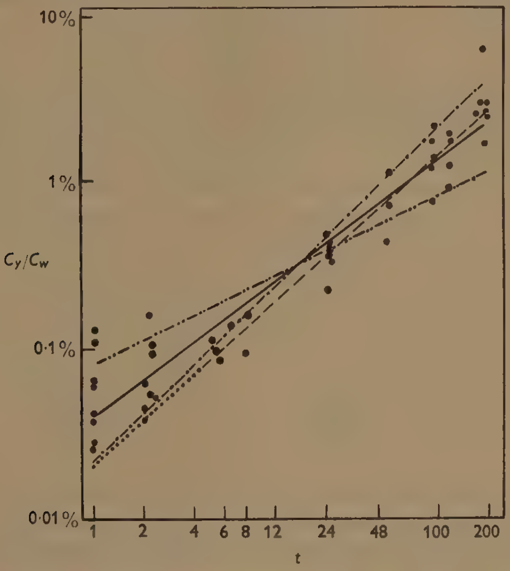

Fig. 1. C_y/C_w: ratio of average concentration of ^{82}Br in yolk to that in white; t: time of penetration in hours; ●: experimental values; —: regression line fitted to all points; ––: regression line fitted to all points after 5 hr.; —·—·: slope corresponding to resistance to diffusion concentrated in external membrane of yolk; —··—··: slope corresponding to resistance to diffusion throughout mass of yolk.

If this is the correct interpretation the amount of ^{82}Br found in the yolk in the first few hours is higher than would be predicted. The excess could result either from a more rapid penetration during the first hour or from contamination by the white while the egg is being frozen or the yolk separated. If contamination is the cause the ratio C_y/C_w should be independent of the experimental time when this is sufficiently short. This ratio was determined for the three eggs where the inner surface of the white was analysed after 5 and 10 min. and lay between 0·01 and 0·02% significantly below the range of its values at 1 hr. This indicates that the contamination is probably no more than 0·02 % and the concentration in the yolk is rising in the first few hours of the experiment. As this rise is not at the rate determined by the resistive membrane it suggests that there is a layer of yolk either outside or actually forming this membrane into which the ^{82}Br can first diffuse. The vitelline membrane remains attached to the white so it could not be the resistive membrane in question which must lie in the yolk itself and will not be morphologically distinguishable.

The outer layer of the yolk into which diffusion takes place freely could, perhaps, be identified with the film of white yolk which can sometimes be seen on its surface.

Distribution of ^{82}Br *in the yolk*

The central mass of white yolk communicates with the outer layer by a narrow channel which widens out below the germinal disk. It is possible that there could be a more rapid entry of ^{82}Br at this point than elsewhere on the surface of the yolk, and it was hoped to investigate this by dividing the frozen yolk and determining whether there was a higher radioactivity in the part containing the germinal disk.

Table 1

Time (hr.)	Vol. yolk (ml.)	Wt. outer layer (g.)	Activity relative to white		Total ^{82}Br, Outer layer/ Whole yolk
			Outer layer	Remainder	
120	15	0·303	0·26	0·014	0·27
120	15	0·423	0·10	0·0063	0·30
120	20	0·490	0·17	0·011	0·27
173	17	0·61	0·22	0·0050	0·63
200	15	0·436	0·50	0·056	0·20

Unfortunately, some tests showed that any difference between the two halves, which could be most marked in the first few hours, could not have been easily distinguished from the variations resulting from the considerable inequalities in the ^{82}Br concentration in the white over the surface of the yolk. However, the penetration by this route is not likely to be very significant, since the germinal disk, 2 mm. in diameter, occupies less than a thousandth part of the superficial area of the yolk and the channel below it is even narrower.

The superficial layer of some frozen yolks was removed in thin slices with a scalpel and collected, weighed, and analysed separately. The results (see Table 1), though few, show unmistakably that there is a far greater concentration of ^{82}Br in the outermost layer than would have resulted if the ion could have diffused freely into the mass of the yolk. It can be seen that after 120 hr. the outermost 0·4 g. of yolk, corresponding to a layer about 0·1 mm. thick, contains nearly one-third of the total ^{82}Br which has penetrated.

This excess cannot come from the white while the egg is being frozen, or the concentration in the yolk would be nearly independent of the experimental time. It is most improbable that it is brought from the mass of the yolk to its surface by freezing since if there was any crystallizing out of salts it would be expected at the yolk centre which freezes last; moreover, it has been shown previously that on freezing in a similar manner the vitreous body of the eye, a more fluid structure, no concentration of salt occurs (Davson, Duke-Elder, Maurice, Ross & Woodin, 1949). Finally, it is hardly credible that the excess results from Br ions becoming chemically bound on entering the yolk.

There must therefore be a resistance to the diffusion of the ion into the mass of the yolk itself. This would seem to be incompatible with the finding that the rate of penetration of ^{82}Br does not decrease with time. However, an exact treatment showed that the internal resistance to diffusion necessary to cause the distribution shown in Table 1 would be small compared to that in the external membrane over the experimental period considered. As a result, the theoretical straight line of slope 1 drawn on Fig. 1 would be modified to an almost straight line of slope 0·95 and would be in closer agreement with the experimental points.

DISCUSSION

The experimental evidence presented here is seen to suggest that the resistance to the diffusion of ^{82}Br is partly in a layer lying in the surface of the yolk and partly in the mass of the yolk itself. It is not, however, capable of deciding what is the nature of this internal resistance.

More evidence is available on this point from an investigation carried out on the electrical properties of the egg (Maurice, 1952). This showed that in the intact state the conductivity of the yolk was very low, both measured in a direction parallel to its surface and at right angles to it. When the yolk was separated and broken up its conductivity was about 40 times greater. This increase is difficult to explain other than by supposing the intact yolk is divided up into compartments by a system of very impermeable but fragile walls.

This structure is compatible with the results of this paper as it provides both a resistance to diffusion within the yolk itself and a limiting resistive membrane corresponding to the outer walls of the most exterior layer of compartments. From an exact treatment of diffusion in this structure it was found that if the inner walls of the compartments had the same permeability to ^{82}Br as the outer ones, i.e. K as derived on page 577, the compartment diameter would have to be about 0·3 mm. to explain the results of Table 1.

The studies of other workers on the permeability of the egg media generally support the view that there is a high resistance to the penetration of ions into the yolk but provide no evidence as to its nature. The only investigations made in conditions comparable to those reported here are those of Hevesy, Levi & Rebbe (1938) and Capraro & Fornaroli (1939). The first named found in one instance the concentration of $^{32}PO_4$ in the yolk to be 3% of that in the white 5 days after its injection into the latter, a figure rather above the average for ^{82}Br. The Italian workers found from two eggs that 24 hr. after the injection of lactic acid under the shell its concentration in the white had increased on the average by 24 mM. and that in the yolk by 3·55 mM. giving a concentration ratio of 15%, 35 times greater than that of ^{82}Br. In neither investigation is it stated at what temperature the eggs were kept and with so few cases no certain comparison can be made of the rates of penetration of these ions with that of ^{82}Br.

The difference in osmotic pressure between yolk and white has drawn much attention to the exchange of water between the two. By injecting heavy water into the white Hevesy *et al.* (1938) were able to show that it distributed itself rapidly throughout the egg, so that after 5 days water from the yolk was only 10% less rich in it than water from the white. If the osmotic difference between yolk and white is abolished by a net flow of water this takes place more slowly than the diffusion of heavy water would indicate, since Smith & Shepherd (1931) showed that the two phases moved half way to osmotic equilibrium in about 30 days if the egg was stored at 0° C., and 12 days at 18° C. Needham (1931*b*) measured the rate at which equilibrium was reached when yolk and white were separated by an isolated vitelline membrane or one of collodion. The freezing-point difference was reduced by half nearly 20 times more rapidly than in the intact egg, allowance being made for the different areas of membrane and volumes of yolk in the two cases. This suggests that the resistance to the diffusion of water in the intact egg also lies in the structure of the yolk. In accordance with this Smith & Shepherd (1931) showed that when a yolk is placed in diluted white, deposits of fluid hypotonic to the yolk are found under the vitelline membrane.

In many investigations the white has been replaced by water or by a fluid of artificial composition and probably some of the effects observed have resulted from precipitation of the globulin or other structural changes in the yolk. An instance is the interesting finding of Orrù (1939) that water entered the yolk rapidly when it was placed in a sugar solution but not when in an isotonic solution of a salt.

SUMMARY

1. ^{82}Br was mixed with the white of hen's eggs maintained at 1° C., and its concentration in the yolk and white measured after experimental periods ranging from 5 min. to 200 hr. In some cases the distribution of ^{82}Br in the yolk was determined.

2. From 5 hr. onwards the concentration in the yolk increased at a constant rate reaching on the average 2·5% of that in the white after 200 hr. This suggested that the penetration was opposed by an external membrane of low permeability (7×10^{-5} cm./hr.).

3. Before 5 hr. the amount of ^{82}Br in the yolk was in excess of that predicted by this mechanism. This did not appear to result from contamination from the white and suggested that there was a layer of yolk external to the resistive external membrane and therefore this was not the vitelline membrane.

4. Even after 100–200 hr. most of the ^{82}Br which had penetrated remained in the superficial layers of the yolk. This showed that there was also a resistance to its penetration in the mass of the yolk itself.

5. The bearing of this evidence on the structure of the yolk is discussed.

This work was carried out by one of us (D.M.M.) during the tenure of a scholarship granted by the Italian Government. He would like to thank Prof. S. Visco for the hospitality of his Institute.

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SOME TABLES FOR THE IONIC COMPOSITION OF SEA WATER

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(*Received* 11 *January* 1954)

There exists a considerable and increasing interest in the ionic composition of the various tissues and body fluids of a wide variety of animals. During the course of such investigations a knowledge of the ionic composition of the natural environment from which the animals were taken is often required. Routine determinations of salinity are usually made in a Marine Biological Laboratory, but the concept of salinity (or chlorinity), of fundamental importance in oceanography, is somewhat artificial and confusion may be felt by those not familiar with the concept.

The object of this paper is to make available a set of tables dealing with the composition of sea water. The preparation of these tables involves the use of the salinity concept, which is first briefly discussed.

A number of physical properties of water depend upon the two variables, temperature and pressure; but in the case of sea water, a third variable, the salt content, has to be considered. It is in its effect upon the physical properties (particularly density) and as an indicator of physical processes (such as evaporation) that the salt content of sea water is of importance in oceanography. The ionic composition is of less interest. Because of its complex composition the total quantity of dissolved solids in any given sample of sea water is difficult to determine by direct analysis. Reproducible results are not obtained by evaporation methods. The difficulties have been avoided by an arbitrary definition of the total salt content, the so-called salinity. Salinity (S‰) was originally defined as the weight in grams (*in vacuo*) of the solids that can be obtained from 1 kg. of sea water (likewise measured *in vacuo*), when all the carbonate has been converted to oxide, the bromine and iodine replaced by chlorine, all organic matter oxidized and the remainder dried at 480° C. to constant weight (Forch, Knudsen & Sörensen, 1902).

Dittmar (1884) established that the ratio between the concentrations of the major ions, in ocean water unaffected by land drainage, was constant and independent of the total salt content; the estimation of a single constituent will, therefore, suffice to determine the total salt content. The major 'constituent' most easily determined is the silver-precipitating halides, which can be determined by titration with silver nitrate and potassium chromate as indicator. Chlorinity (Cl‰) is defined as the weight in grams (*in vacuo*) of the chlorides obtained in 1 kg. of sea water (likewise measured *in vacuo*) when all the bromides and iodides have been replaced by chlorides. To the above definition must be added the proviso that the reference standard is Normal Water (see below), and the titration result be calculated using Knudsen's Hydrographical Tables (1901). The following equation expresses the relation between salinity and chlorinity:

$$S\text{‰} = 0{\cdot}03 + 1{\cdot}805 \times \text{Cl}\text{‰}. \qquad (1)$$

This definition of chlorinity introduces a chlorine equivalent which is dependent upon the atomic weights of the elements present in the standard solution. In order that the apparent chlorinity of the oceans shall not vary as revisions of atomic weights affect the appropriate gravimetric factors, it is essential to maintain an international standard for chlorinity which is independent of atomic weight changes. This has been effected in the past by standardization of all solutions against the so-called Normal Water (Eau de mer normale) which has its chlorinity adjusted to about 19·4‰ and which is accurately standardized either directly or indirectly against the 1902 standard. This standard sea water has been prepared and distributed by the Hydrographic Laboratories at Copenhagen. More recently, Jacobsen & Knudsen (1940) have established a permanent standard for chlorinity by standardizing Normal Water against the mass of silver required completely to precipitate the halogens in 1 kg. of such water. On the basis of this work a new definition of salinity may be given; the number giving the chlorinity in grams per kilogram of sea water sample is identical with the number giving the mass in grams of 'atomic weight silver' just necessary to precipitate the halogens in 0·3285233 kg. of sea-water sample. This new definition makes the chlorinity independent of any atomic weight changes and the empirically established relations between salinity and chlorinity remain valid.

We may now turn to the ionic composition. The best values of the ionic ratios have been selected by Lyman & Fleming (1940) after a critical examination of published work; they are given below, together with the weights per kilogram of sea water for sea water with a chlorinity of 19·00‰.

Ion...	Cl	Br	SO_4	HCO_3	Ca
Ratio to chlorinity	0·99894	0·00340	0·1394	0·00735	0·02106
	Mg	K	Sr	Na	H_3BO_3
	0·06695	0·0200	0·000702	0·5556	0·00137
	Cl	Br	SO_4	HCO_3	Ca
Grams. per kilogram	18·9799	0·0646	2·6486	0·1397	0·4001
	Mg	K	Sr	Na	H_3BO_3
	1·2720	0·3800	0·0133	10·5561	0·0260

The total salt content calculated from these values is 34·4816 g./kg.; converting the bromide to chloride and the bicarbonate to oxide, this gives for the salinity as defined above 34·3243‰ as compared with 34·325‰ given by Knudsen's equation (1).

Equation (1) implies that water having negligible chlorinity still contains other anions, carbonate and sulphate, and the ratios of the major constituents to chlorinity must, therefore, be a function of chlorinity. The major constituents whose ratios vary significantly with chlorinity are calcium, magnesium, carbonate and sulphate.

Lyman & Fleming (1940) give equations for the relation between chlorinity and these four constituents, as follows:

$$SO_4 = 0{\cdot}0061 + 0{\cdot}1391\,Cl, \quad (2)$$

$$Ca = 0{\cdot}0154 + 0{\cdot}02025\,Cl, \quad (3)$$

$$Mg = 0{\cdot}0020 + 0{\cdot}06684\,Cl, \quad (4)$$

$$HCO_3 = 0{\cdot}0493 + 0{\cdot}004755\,Cl. \quad (5)$$

Total salts (as distinguished from salinity)

$$= 0{\cdot}073 + 1{\cdot}8110\,Cl. \quad (6)$$

Using these equations for the four ions indicated and assuming the ratios to chlorinity of all the other components are constant and are given by the values in Table 1 (even if slightly incorrect, this will introduce no significant discrepancy) the composition of sea water equivalent to any chlorinity (determined in the standard way using Knudsen's Hydrographical Tables) can be calculated. Knowing the density of the sea water at the given chlorinity the values can be converted to a volume basis.

The following tables have been prepared:

Table 1. Grams per kilogram of sea water for chlorinity 1–23‰ (S = 1·84–41·55‰): calculated from the best available ratios of ions to chlorinity (Lyman & Fleming, 1940).

Table 2. Grams per litre (20° C.) of sea water for chlorinity 1–23‰ (S = 1·84–41·55‰): calculated from Table 1 and the densities obtained from Knudsen's Tables.

Table 3. Millimoles (milligram-ions) per kilogram of sea water for chlorinity 1–23‰ (S = 1·84–41·55‰): calculated from Table 1, and 1940 values for atomic weights.

Table 4. Millimoles (milligram-ions) per litre (20° C.) of sea water (molarity) for chlorinity 1–23‰ (S = 1·84–41·55‰): calculated from Table 2 and the 1940 values for atomic weights. *Note*: the milliequivalents per kilogram or per litre (20° C.) are readily obtained from Tables 3 and 4 by multiplying the values by the valency of the particular ion.

Table 5. Grams per kilogram of water for chlorinity 1–23‰ (S = 1·84–41·55‰): calculated from Table 1 and the values of total salt content from equation (6) (see text).

Table 6. Millimoles (milligram-ions) per kilogram of water (molality) for chlorinity 1–23‰ (S = 1·84–41·55‰): calculated from Table 5 and the 1940 values for atomic weights. *Note*: milliequivalents can be calculated from Table 6 by multiplying the values by the valency of the particular ion.

Table 7. A table of isosmotic solutions was originally prepared from the published values of the depression of freezing-point for a number of solutes and for sea water (Knudsen, 1903). Recently, Prof. Robinson has made more direct comparisons by the isopiestic method (Robinson, 1954) and, through the courtesy of the Director of the Marine Biological Association of the United Kingdom, Prof. Robinson's table was made available prior to publication, and it is reproduced here.

Table 1. *Grams per kilogram of sea water for chlorinity* 1–23‰
(S=1·84–41·55‰)

Cl‰	S‰	Cl	Br	SO_4	HCO_3	Ca	Mg	K	Sr	Na	H_3BO_3
1	1·84	0·9989	0·0034	0·1452	0·05406	0·03565	0·06884	0·0200	0·000702	0·5556	0·00137
2	3·64	1·9979	0·0068	0·2843	0·05881	0·05590	0·1357	0·0400	0·001404	1·1112	0·00274
3	5·45	2·9968	0·0102	0·4234	0·06357	0·07615	0·2025	0·0600	0·002106	1·6668	0·00411
4	7·25	3·9958	0·0136	0·5625	0·06832	0·09640	0·2694	0·0800	0·002808	2·2224	0·00548
5	9·06	4·9947	0·0170	0·7016	0·07308	0·1167	0·3362	0·1000	0·003510	2·7780	0·00685
6	10·86	5·9936	0·0204	0·8407	0·07783	0·1369	0·4030	0·1200	0·004212	3·3336	0·00822
7	12·67	6·9926	0·0238	0·9798	0·08259	0·1572	0·4699	0·1400	0·004914	3·8892	0·00959
8	14·47	7·9915	0·0272	1·1189	0·08734	0·1774	0·5367	0·1600	0·005616	4·4448	0·01096
9	16·28	8·9905	0·0306	1·2580	0·09210	0·1977	0·6036	0·1800	0·006318	5·0004	0·01233
10	18·08	9·9894	0·0340	1·3971	0·09685	0·2179	0·6704	0·2000	0·007020	5·5560	0·01370
11	19·89	10·9883	0·0374	1·5362	0·1016	0·2382	0·7372	0·2200	0·007722	6·1116	0·01507
12	21·69	11·9873	0·0408	1·6753	0·1064	0·2584	0·8041	0·2400	0·008424	6·6672	0·01644
13	23·50	12·9862	0·0442	1·8144	0·1111	0·2787	0·8709	0·2600	0·009126	7·2228	0·01781
14	25·30	13·9852	0·0476	1·9535	0·1159	0·2989	0·9378	0·2800	0·009828	7·7784	0·01918
15	27·11	14·9841	0·0510	2·0926	0·1206	0·3192	1·0046	0·3000	0·01053	8·3340	0·02055
16	28·91	15·9830	0·0544	2·2317	0·1254	0·3394	1·0714	0·3200	0·01123	8·8896	0·02192
17	30·72	16·9820	0·0578	2·3708	0·1301	0·3597	1·1383	0·3400	0·01193	9·4452	0·02329
18	32·52	17·9809	0·0612	2·5099	0·1349	0·3799	1·2051	0·3600	0·01264	10·0008	0·02466
19	34·33	18·9799	0·0646	2·6490	0·1396	0·4002	1·2720	0·3800	0·01334	10·5564	0·02603
20	36·13	19·9788	0·0680	2·7881	0·1444	0·4204	1·3388	0·4000	0·01404	11·1120	0·02740
21	37·94	20·9777	0·0714	2·9272	0·1492	0·4407	1·4056	0·4200	0·01474	11·6676	0·02877
22	39·74	21·9767	0·0748	3·0663	0·1539	0·4609	1·4725	0·4400	0·01544	12·2232	0·03014
23	41·55	22·9756	0·0782	3·2054	0·1587	0·4812	1·5393	0·4600	0·01615	12·7788	0·03151

Table 2. *Grams per litre* (20° C) *of sea water for chlorinity* 1–23‰
(S=1·84–41·55‰)

Cl‰	S‰	Cl	Br	SO_4	HCO_3	Ca	Mg	K	Sr	Na	H_3BO_3
1	1·84	0·9986	0·003399	0·1452	0·05404	0·03564	0·06882	0·01999	0·0007018	0·5554	0·001370
2	3·64	2·0000	0·006807	0·2846	0·05887	0·05596	0·1358	0·04004	0·001405	1·1124	0·002743
3	5·45	3·0040	0·01022	0·4244	0·06372	0·07633	0·2030	0·06014	0·002111	1·6708	0·004120
4	7·25	4·0108	0·01365	0·5646	0·06858	0·09676	0·2704	0·08030	0·002819	2·2308	0·005501
5	9·06	5·0204	0·01709	0·7052	0·07345	0·1172	0·3379	0·1005	0·003528	2·7923	0·006885
6	10·86	6·0327	0·02053	0·8462	0·07834	0·1378	0·4057	0·1208	0·004239	3·3553	0·008274
7	12·67	7·0476	0·02399	0·9875	0·08323	0·1584	0·4736	0·1411	0·004953	3·9198	0·009665
8	14·47	8·0654	0·02745	1·1292	0·08815	0·1790	0·5417	0·1615	0·005668	4·4859	0·01106
9	16·28	9·0858	0·03092	1·2713	0·09307	0·1997	0·6100	0·1819	0·006385	5·0534	0·01246
10	18·08	10·1089	0·03441	1·4138	0·09801	0·2205	0·6784	0·2024	0·007104	5·6225	0·01386
11	19·89	11·1348	0·03790	1·5567	0·1030	0·2413	0·7471	0·2229	0·007825	6·1930	0·01527
12	21·69	12·1634	0·04140	1·6999	0·1079	0·2622	0·8159	0·2435	0·008548	6·7651	0·01668
13	23·50	13·1947	0·04491	1·8435	0·1129	0·2831	0·8849	0·2642	0·009273	7·3388	0·01810
14	25·30	14·2288	0·04843	1·9875	0·1179	0·3041	0·9541	0·2849	0·009999	7·9139	0·01951
15	27·11	15·2656	0·05196	2·1319	0·1229	0·3251	1·0235	0·3056	0·01073	8·4906	0·02094
16	28·91	16·3051	0·05550	2·2767	0·1279	0·3462	1·0930	0·3264	0·01146	9·0687	0·02236
17	30·72	17·3476	0·05904	2·4218	0·1329	0·3674	1·1628	0·3473	0·01219	9·6485	0·02379
18	32·52	18·3926	0·06260	2·5674	0·1380	0·3886	1·2327	0·3682	0·01293	10·2298	0·02522
19	34·33	19·4404	0·06617	2·7133	0·1430	0·4099	1·3028	0·3892	0·01366	10·8126	0·02666
20	36·13	20·4911	0·06974	2·8596	0·1481	0·4312	1·3731	0·4103	0·01440	11·3970	0·02810
21	37·94	21·5444	0·07333	3·0063	0·1532	0·4526	1·4436	0·4313	0·01514	11·9828	0·02955
22	39·74	22·6007	0·07692	3·1534	0·1583	0·4740	1·5143	0·4525	0·01588	12·5703	0·03100
23	41·55	23·6597	0·08053	3·3008	0·1634	0·4955	1·5852	0·4737	0·01663	13·1593	0·03245

Table 3. *Millimoles (milligram-ions) per kilogram of sea water for chlorinity* 1–23‰ (S=1·84–41·55‰)

Cl‰	S‰	Cl	Br	SO_4	HCO_3	Ca	Mg	K	Sr	Na	H_3BO_3
1	1·84	28·17	0·04254	1·512	0·8859	0·8895	2·831	0·5116	0·008011	24·16	0·02215
2	3·64	56·35	0·08509	2·960	0·9638	1·395	5·579	1·023	0·01602	48·32	0·04431
3	5·45	84·52	0·1276	4·408	1·042	1·900	8·327	1·535	0·02403	72·48	0·06646
4	7·25	112·7	0·1702	5·856	1·120	2·405	11·08	2·046	0·03204	96·64	0·08861
5	9·06	140·9	0·2127	7·304	1·198	2·910	13·82	2·558	0·04005	120·8	0·1108
6	10·86	169·0	0·2553	8·752	1·276	3·416	16·57	3·069	0·04807	145·0	0·1329
7	12·67	197·2	0·2978	10·20	1·354	3·921	19·32	3·581	0·05608	169·1	0·1551
8	14·47	225·4	0·3404	11·65	1·431	4·426	22·07	4·093	0·06409	193·3	0·1772
9	16·28	253·6	0·3829	13·10	1·509	4·931	24·82	4·604	0·07210	217·4	0·1994
10	18·08	281·7	0·4254	14·54	1·587	5·437	27·57	5·116	0·08011	241·6	0·2215
11	19·89	309·9	0·4680	15·99	1·665	5·942	30·31	5·627	0·08812	265·8	0·2437
12	21·69	338·1	0·5105	17·44	1·743	6·447	33·06	6·139	0·09613	289·9	0·2658
13	23·50	366·3	0·5531	18·89	1·821	6·952	35·81	6·650	0·1041	314·1	0·2880
14	25·30	394·4	0·5956	20·34	1·899	7·458	38·56	7·162	0·1122	338·2	0·3101
15	27·11	422·6	0·6382	21·78	1·977	7·963	41·31	7·673	0·1202	362·4	0·3323
16	28·91	450·8	0·6807	23·23	2·055	8·468	44·06	8·185	0·1282	386·6	0·3544
17	30·72	478·9	0·7233	24·68	2·133	8·973	46·80	8·697	0·1362	410·7	0·3766
18	32·52	507·1	0·7658	26·13	2·211	9·479	49·55	9·208	0·1442	434·9	0·3987
19	34·33	535·3	0·8083	27·58	2·289	9·984	52·30	9·720	0·1522	459·0	0·4209
20	36·13	563·5	0·8509	29·02	2·367	10·49	55·05	10·23	0·1602	483·2	0·4431
21	37·94	591·6	0·8934	30·47	2·444	10·99	57·80	10·74	0·1682	507·4	0·4652
22	39·74	619·8	0·9360	31·92	2·522	11·50	60·55	11·25	0·1762	531·5	0·4874
23	41·55	648·0	0·9785	33·37	2·600	12·00	63·29	11·77	0·1843	555·7	0·5095

Table 4. *Millimoles (milligram-ions) per litre* (20° C.) *of sea water* (*molarity*) *for chlorinity* 1–23‰ (S=1·84–41·55‰)

Cl‰	S‰	Cl	Br	SO_4	HCO_3	Ca	Mg	K	Sr	Na	H_3BO_3
1	1·84	28·16	0·04253	1·511	0·8856	0·8892	2·830	0·5114	0·008008	24·15	0·02215
2	3·64	56·41	0·08518	2·963	0·9648	1·396	5·585	1·024	0·01604	48·37	0·04435
3	5·45	84·72	0·1279	4·418	1·044	1·905	8·347	1·538	0·02409	72·65	0·06662
4	7·25	113·1	0·1708	5·878	1·124	2·414	11·12	2·054	0·03216	97·00	0·08894
5	9·06	141·6	0·2138	7·341	1·204	2·925	13·90	2·571	0·04026	121·4	0·1113
6	10·86	170·1	0·2569	8·809	1·284	3·438	16·68	3·089	0·04838	145·9	0·1338
7	12·67	198·8	0·3002	10·28	1·364	3·952	19·47	3·609	0·05652	170·4	0·1563
8	14·47	227·5	0·3435	11·76	1·445	4·467	22·27	4·130	0·06468	195·1	0·1789
9	16·28	256·2	0·3870	13·23	1·525	4·984	25·08	4·653	0·07286	219·7	0·2015
10	18·08	285·1	0·4305	14·72	1·606	5·502	27·90	5·177	0·08107	244·5	0·2242
11	19·89	314·0	0·4742	16·21	1·687	6·021	30·72	5·702	0·08929	269·3	0·2469
12	21·69	343·0	0·5180	17·70	1·769	6·542	33·55	6·229	0·09754	294·2	0·2697
13	23·50	372·1	0·5620	19·19	1·850	7·064	36·39	6·757	0·1058	319·1	0·2926
14	25·30	401·3	0·6060	20·69	1·932	7·588	39·23	7·287	0·1141	344·1	0·3155
15	27·11	430·5	0·6502	22·19	2·014	8·112	42·08	7·818	0·1224	369·2	0·3385
16	28·91	459·9	0·6944	23·70	2·096	8·639	44·94	8·350	0·1308	394·3	0·3616
17	30·72	489·3	0·7388	25·21	2·179	9·167	47·81	8·884	0·1391	419·6	0·3847
18	32·52	518·7	0·7833	26·73	2·261	9·696	50·69	9·419	0·1475	444·8	0·4079
19	34·33	548·3	0·8280	28·25	2·344	10·23	53·57	9·956	0·1559	470·2	0·4311
20	36·13	577·9	0·8727	29·77	2·427	10·76	56·46	10·49	0·1643	495·6	0·4544
21	37·94	607·6	0·9176	31·30	2·511	11·29	59·36	11·03	0·1728	521·1	0·4778
22	39·74	637·4	0·9626	32·83	2·594	11·83	62·27	11·57	0·1812	546·6	0·5012
23	41·55	667·3	1·008	34·36	2·678	12·36	65·18	12·12	0·1897	572·2	0·5247

Table 5. *Grams per kilogram of water for chlorinity* 1–23‰ (S = 1·84–41·55‰)

‰	S‰	Cl	Br	SO_4	HCO_3	Ca	Mg	K	Sr	Na	H_3BO_3
	1·84	1·0008	0·003406	0·1455	0·05416	0·03572	0·06897	0·02004	0·0007033	0·5566	0·001373
	3·64	2·0053	0·006825	0·2854	0·05903	0·05611	0·1362	0·04015	0·001409	1·1153	0·002750
	5·45	3·0134	0·01026	0·4257	0·06392	0·07657	0·2036	0·06033	0·002118	1·6760	0·004133
	7·25	4·0252	0·01370	0·5666	0·06882	0·09711	0·2713	0·08059	0·002829	2·2388	0·005520
	9·06	5·0407	0·01716	0·7081	0·07375	0·1177	0·3393	0·1009	0·003542	2·8036	0·006913
	10·86	6·0599	0·02063	0·8500	0·07869	0·1384	0·4075	0·1213	0·004259	3·3705	0·008311
	12·67	7·0829	0·02411	0·9925	0·08365	0·1592	0·4759	0·1418	0·004977	3·9394	0·009714
	14·47	8·1096	0·02760	1·1354	0·08863	0·1800	0·5447	0·1624	0·005699	4·5105	0·01112
	16·28	9·1401	0·03111	1·2789	0·09363	0·2009	0·6136	0·1830	0·006423	5·0836	0·01254
	18·08	10·1744	0·03463	1·4230	0·09864	0·2219	0·6828	0·2037	0·007150	5·6589	0·01395
	19·89	11·2125	0·03816	1·5675	0·1037	0·2430	0·7523	0·2245	0·007880	6·2363	0·01538
	21·69	12·2545	0·04171	1·7126	0·1087	0·2642	0·8220	0·2453	0·008612	6·8158	0·01681
	23·50	13·3003	0·04527	1·8583	0·1138	0·2854	0·8920	0·2663	0·009347	7·3975	0·01824
	25·30	14·3500	0·04884	2·0045	0·1189	0·3067	0·9622	0·2873	0·01008	7·9813	0·01968
	27·11	15·4037	0·05243	2·1512	0·1240	0·3281	1·0327	0·3084	0·01082	8·5674	0·02113
	28·91	16·4612	0·05603	2·2985	0·1291	0·3496	1·1035	0·3296	0·01157	9·1556	0·02258
	30·72	17·5227	0·05964	2·4463	0·1343	0·3711	1·1745	0·3508	0·01231	9·7460	0·02403
	32·52	18·5882	0·06327	2·5947	0·1394	0·3927	1·2458	0·3722	0·01306	10·3386	0·02549
	34·33	19·6577	0·06691	2·7436	0·1446	0·4144	1·3174	0·3936	0·01381	10·9334	0·02696
	36·13	20·7312	0·07056	2·8931	0·1498	0·4362	1·3892	0·4151	0·01457	11·5305	0·02843
	37·94	21·8087	0·07423	3·0432	0·1551	0·4581	1·4613	0·4366	0·01533	12·1298	0·02991
	39·74	22·8903	0·07791	3·1938	0·1603	0·4801	1·5337	0·4583	0·01609	12·7314	0·03139
	41·55	23·9760	0·08161	3·3450	0·1656	0·5021	1·6063	0·4800	0·01685	13·3352	0·03288

Table 6. *Millimoles (milligram-ions) per kilogram of water (molality) for chlorinity* 1–23‰ (S = 1·84–41·55‰)

C‰	S‰	Cl	Br	SO_4	HCO_3	Ca	Mg	K	Sr	Na	H_3BO_3
1	1·84	28·23	0·04262	1·514	0·8876	0·8911	2·836	0·5125	0·008026	24·21	0·02219
2	3·64	56·56	0·08540	2·971	0·9674	1·400	5·600	1·027	0·01608	48·50	0·04447
3	5·45	84·99	0·1283	4·432	1·048	1·910	8·373	1·543	0·02417	72·88	0·06683
4	7·25	113·5	0·1714	5·899	1·128	2·423	11·16	2·061	0·03228	97·35	0·08926
5	9·06	142·1	0·2147	7·371	1·209	2·937	13·95	2·581	0·04042	121·9	0·1118
6	10·86	170·9	0·2581	8·849	1·290	3·453	16·76	3·103	0·04860	146·6	0·1344
7	12·67	199·8	0·3017	10·33	1·371	3·972	19·57	3·627	0·05680	171·3	0·1571
8	14·47	228·7	0·3454	11·82	1·453	4·492	22·40	4·153	0·06503	196·1	0·1798
9	16·28	257·8	0·3893	13·31	1·534	5·013	25·23	4·681	0·07330	221·1	0·2027
10	18·08	287·0	0·4333	14·81	1·617	5·537	28·08	5·210	0·08159	246·1	0·2256
11	19·89	316·2	0·4775	16·32	1·699	6·063	30·93	5·742	0·08992	271·2	0·2486
12	21·69	345·6	0·5219	17·83	1·782	6·591	33·80	6·276	0·09827	296·4	0·2718
13	23·50	375·1	0·5665	19·35	1·865	7·121	36·68	6·811	0·1067	321·7	0·2949
14	25·30	404·7	0·6112	20·87	1·948	7·652	39·57	7·349	0·1151	347·1	0·3182
15	27·11	434·4	0·6560	22·39	2·032	8·186	42·46	7·888	0·1235	372·5	0·3416
16	28·91	464·3	0·7011	23·93	2·116	8·721	45·37	8·430	0·1320	398·1	0·3650
17	30·72	494·2	0·7463	25·47	2·201	9·259	48·29	8·973	0·1405	423·8	0·3886
18	32·52	524·2	0·7917	27·01	2·285	9·799	51·23	9·519	0·1491	449·6	0·4122
19	34·33	554·4	0·8372	28·56	2·370	10·34	54·17	10·07	0·1576	475·4	0·4359
20	36·13	584·7	0·8829	30·12	2·456	10·88	57·12	10·62	0·1663	501·4	0·4597
21	37·94	615·1	0·9288	31·68	2·541	11·43	60·09	11·17	0·1749	527·5	0·4836
22	39·74	645·6	0·9749	33·25	2·627	11·98	63·06	11·72	0·1836	553·6	0·5076
23	41·58	676·2	1·021	34·82	2·714	12·53	66·05	12·28	0·1923	579·9	0·5317

Table 7. *Vapour pressure and osmotic equivalence of sea water at 25° C.*

Cl‰	*R*	NaCl	KCl	$CaCl_2$	$MgCl_2$	$MgSO_4$	Na_2SO_4	Sucrose	Urea	V.P. lowering	Osmotic pressure (atm.)
10	0·02861	0·2861	0·2908	0·2039	0·2005	0·5056	0·2374	0·5065	0·5400	0·00946	12·87
11	0·02869	0·3156	0·3211	0·2240	0·2199	0·5597	0·2643	0·5560	0·5965	0·01042	14·19
12	0·02877	0·3452	0·3516	0·2441	0·2393	0·6138	0·2918	0·6053	0·6534	0·01139	15·51
13	0·02885	0·3751	0·3825	0·2642	0·2588	0·6675	0·3196	0·6546	0·7112	0·01237	16·85
14	0·02893	0·4050	0·4134	0·2841	0·2780	0·7206	0·3477	0·7040	0·7695	0·01334	18·19
15	0·02901	0·4352	0·4447	0·3043	0·2975	0·7738	0·3762	0·7534	0·8285	0·01433	19·55
16	0·02908	0·4653	0·4760	0·3243	0·3165	0·8264	0·4051	0·8025	0·8880	0·01532	20·91
17	0·02916	0·4957	0·5077	0·3445	0·3356	0·8786	0·4347	0·8516	0·9482	0·01631	22·28
18	0·02924	0·5263	0·5397	0·3645	0·3546	0·9300	0·4648	0·9008	1·010	0·01732	23·66
19	0·02932	0·5571	0·5719	0·3845	0·3738	0·9803	0·4954	0·9497	1·071	0·01833	25·06
20	0·02940	0·5880	0·6043	0·4044	0·3929	1·028	0·5264	0·9982	1·133	0·01936	26·47
21	0·02948	0·6191	0·6370	0·4243	0·4122	1·076	0·5578	1·047	1·197	0·02039	27·89
22	0·02956	0·6503	0·6698	0·4440	0·4313	1·123	0·5896	1·095	1·260	0·02142	29·33

The column headed V.P. lowering gives the relative pressure lowering $\Delta p/p^0=(p^0-p)/p^0$, where p is the vapour pressure of the sea water and p^0 is the vapour pressure of pure water. $p^0=23·756$ mm. 25° C. R=M-NaCl/‰ Cl.

The data are not more accurate than warrants five significant figures in the tables, the accuracy varying with the analytical methods involved. In some of the tables more figures are quoted and may be rounded off; in tables of milligram-ions only four significant figures are given.

I wish to thank both Dr L. H. N. Cooper and Dr J. D. Robertson for helpful criticism and for checks on a number of the tables, and the former for drawing attention to Prof. Robinson's work.

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J. Exp. Biol 31
1954

THE RESPIRATORY CURRENTS IN THE TRACHEAL SYSTEM OF THE ADULT HONEY-BEE

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(*Received* 19 *January* 1954)

INTRODUCTION

Interest in the respiration of the bee has been aroused on several occasions in the past, largely because of the disease caused by the infestation of the tracheae of the first thoracic spiracles by the mite *Acarapis woodi* (Rennie).

White (1921) investigated the pathological effects on the bee of blocking the anterior spiracles with wax. He found that such bees lost the power of flight at once and he related this to the similar effects of severe infestation by *A. woodi*.

Morison (1927) put powdered charcoal in the depression in the cuticle close to the aperture of the anterior spiracles and noted that the charcoal was alternately blown away and drawn towards the spiracular opening. Since the powder was only feebly drawn towards the opening Morison concluded that a greater volume of air left the spiracle than entered it, and that there was a circulation of air from the posterior regions of the body of the bee to the anterior spiracle.

Wohlgemuth (1929) separated the abdomen and thorax of the bee with a rubber diaphragm and was able to anaesthetize the insect by putting either the thorax or abdomen into chloroform vapour. He concluded that inspiration took place by both abdominal and thoracic tracheae. He also examined the flow of air near the spiracular openings by holding a needle covered with minute soot particles near the openings and observing the movements of the particles. He finally concluded that the bee inspired through all spiracles during abdominal expansion and that expiration occurred through the thoracic spiracles only.

Sachs (1952) was able to show that individuals of *A. woodi*, when placed on the thorax of the bee, were attracted towards the first spiracle. By supplying an artificial current of air to the first spiracles of the empty thoraces of dead bees he found that the mite was attracted to the opening by intermittent puffs of air of about 120 per minute.

Connell & Glynne-Jones (1953) examined the respiratory activity of bees by (*a*) using a modification of the apparatus used by Fraenkel (1932), (*b*) high-speed photography, and (*c*) observing the entry of minute dust particles into the spiracles. They concluded that both thoracic and abdominal spiracles serve for expiration and inspiration, and that a current of air flowing from abdomen to thorax or vice versa was unlikely.

The following paper describes further observations upon the respiratory currents in the tracheae of bees. It has been found that the air circulation is rather more complex than has previously been supposed.

METHODS

The activity of the propodeal ('third thoracic') spiracles was observed directly, with the bee lightly clamped under a binocular microscope. The activity of the other spiracles cannot be seen. The thoracic spiracles are hidden under folds of the cuticle, and the abdominal spiracles open into cavities which then lead to a passive external opening. (Snodgrass (1925) and Wohlgemuth (1929) give full descriptions of the anatomy of the spiracles.)

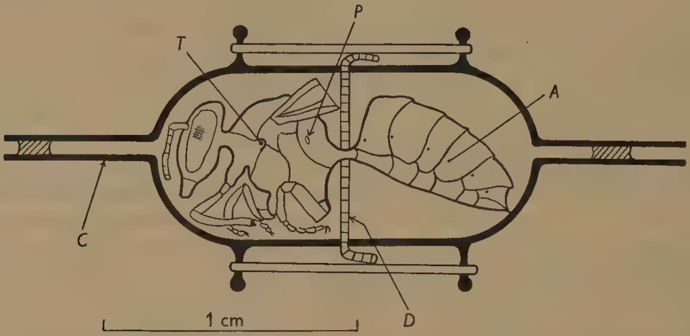

Fig. 1. Diagram of the apparatus used with the bee in the gas chamber. *C*, capillary tube leading from main chamber; *D*, rubber diaphragm; *A*, one of the six pairs of external openings of the abdominal spiracles; *P*, propodeal ('third thoracic') spiracle; *T*, lobe of body wall shielding first thoracic spiracle.

Fraenkel's technique (1932) was used to observe the air currents passing through the body of the bee. A gas chamber was made from glass tubing of 6 mm. internal diameter. The chamber was about 2 cm. long and both ends were drawn out into fine capillaries of about 0·5 mm. internal diameter. A hole was burned into a thin rubber diaphragm and the diameter of the hole was made approximately the same as the diameter of the petiole of the bee. The bee was fixed in the diaphragm and a small drop of water was spread on to the petiole, thereby sealing the joint between it and the rubber. The rubber was held firmly between the ground ends of the two halves of the chamber with the thorax and head of the bee in one half and the abdomen in the other half. Drops of water were introduced into each capillary tube. The wings of the bee were clipped short before it was placed in the chamber (Fig. 1).

For some experiments the first thoracic spiracles or the propodeal spiracles were blocked with beeswax in the following manner. The bee was anaesthetized with CO_2 and clamped lightly under a binocular microscope; a piece of wax was held on the spiracle with a fine needle and the needle was then touched with a hot wire. The wax melted over the spiracle and the needle was quickly withdrawn.

The second thoracic spiracles were ignored since their openings are exceedingly minute relative to those of the other spiracles.

RESULTS

A. *The action of the propodeal spiracles*

Usually the spiracles were closed, but occasionally they opened and closed with a rhythm which appeared to be synchronous with abdominal pumping; such movements were rapid and of brief duration.

The spiracular mechanism was very sensitive to carbon dioxide; the direction of a stream of carbon dioxide on to a bee whose third spiracle was closed caused the latter to begin rhythmical movements immediately. By this means it was established that the spiracle was open when the abdomen was contracting and closed when the abdomen was expanding. Exposure to a high CO_2 concentration anaesthetized the bee and the spiracle again became permanently closed whilst the bee was immobile.

B. *The movement of air in the chamber*

(*a*) *Normal bees*

(i) *In normal air*. The drops of water in the capillary tubes began oscillating as soon as the bee was enclosed in the chamber. Their movements were approximately 1 cm. each way, synchronized and in the same direction. The drop in the abdominal capillary tube approached the bee as the abdomen contracted and receded as the abdomen expanded. The drop in the thoracic tube receded from the bee as the abdomen contracted and approached as the abdomen expanded. The frequency of this movement was between 100 and 200 cyc./min.

The centre of oscillation of the drops usually moved slowly along the tubes (about 5 mm. in about 10–30 sec.) as a result of the transference of air from one half of the chamber to the other. The flow was usually from thorax to abdomen but the direction of this slow airstream was variable.

(ii) *In air plus* CO_2. A bee was allowed to feed on strong syrup and was then placed in the chamber. It was allowed to settle down for some minutes with the two halves of the chamber left ajar. The halves were then closed upon each other. Strong oscillating movements of the drops occurred with a superimposed migration showing a slow air flow from thorax to abdomen.

CO_2 was then blown into both sides of the chamber; the slow air flow immediately reversed direction but the superimposed oscillations continued. The chamber was then opened, aired for a minute or two, and then closed; the air flow now returned to its original direction. This experiment was repeated several times. The drop of water in the abdominal capillary tube was then removed and the bee was left undisturbed in the chamber; the drop of water in the thoracic tube was drawn towards the head and eventually it spread out and burst as the tube widened into the chamber. The water then ran back into the narrow tube and repeated the process. The bee was left overnight and next day the drop of water was still moving towards the head and bursting. CO_2 was then blown into the chamber and again there was an immediate and definite reversal of this air flow.

From the results of the experiments described above it appears that a tidal flow of air normally passes in and out of the thoracic spiracles and this flow is caused by the pumping movements of the abdomen.

The superimposed slow current of air was undoubtedly regulated by the activities of the spiracles. In air plus CO_2 the abdominal spiracles must have been relatively closed compared with the thoracic spiracles when the abdomen was contracting and relatively open when the abdomen was expanding. Thus a movement of air from abdomen to thorax was created.

The observed activity of the propodeal spiracle in air plus CO_2 was consistent with this interpretation. However, in air plus CO_2, air must have passed into the thoracic spiracles to some extent when the abdomen was expanding, otherwise there would have been no oscillation of the water drops.

(*b*) *Bees with blocked propodeal spiracles*

(i) *In normal air*. The behaviour of the water drops in the capillary tubes seemed to be exactly as before. Strong oscillation of the same amplitude occurred and overall migration of the drops was usually from thorax to abdomen but occasionally this was reversed.

(ii) *In air plus* CO_2. The air current was now rapid and its direction was consistently from thorax to abdomen, but the strong oscillations of the drops continued.

It now seemed that the first thoracic spiracles were also affected by CO_2, but their opening and closing movements, although still synchronized with abdominal pumping, were in opposition to those of the propodeal spiracles. Thus they were open when the abdomen was expanding and were closed relative to the abdominal spiracles when the abdomen was contracting in air and CO_2, thereby creating a flow of air from thorax to abdomen.

They could not have closed completely, however, since the oscillation of the water drops continued and some air must therefore have been passing out of the partly closed spiracles when the abdomen was contracting.

(*c*) *Bees with blocked first thoracic spiracles*

(i) *In normal air*. There was hardly any movement of the drops, the oscillation being no more than 1 or 2 mm., with no detectable directed airflow.

(ii) *In air plus* CO_2. There was an extremely rapid movement of air from the abdominal chamber to the thoracic one and there was no oscillation. The drops of water moved along in jerks synchronous with the contraction of the abdomen.

Since the anterior spiracles were blocked and the propodeal spiracles were closed there was neither a tidal oscillation nor an air stream in normal air.

In air plus CO_2 air was pumped efficiently from the abdomen through the propodeal spiracles. The lack of oscillation in normal air and air plus CO_2 meant that no air could pass into the propodeal spiracle (or the second thoracic spiracle) as the abdomen expanded. The tidal oscillation occurring under normal conditions must therefore take place via the first thoracic spiracle.

DISCUSSION

It was concluded from these results that the inactive bee inhales and exhales via the first thoracic spiracles, the air movement being created by the pumping movements of the abdomen. The flying bee probably changes to the other condition when the spiracles come into action under the stimulus of metabolic CO_2 production. Air would then be inhaled at the first spiracle and largely exhaled via the propodeal spiracle. Thus a rapid flow of fresh air would pass through the thorax of the insect providing thorough ventilation in a region of relatively high carbon dioxide production.*

The inability of bees to fly when heavily infested with *Acarapis woodi* in the tracheae leading from the first spiracles, or when the latter are artificially blocked, is therefore understandable.

Movement of air through the abdominal spiracles is slight under low CO_2 tensions; but a current of air probably flows into them and out of the propodeal spiracle under high CO_2 tensions.

SUMMARY

1. The propodeal spiracles of the honey-bee remain closed in normal air but in air plus CO_2 they open as the abdomen contracts, and close as the abdomen expands.

2. The first thoracic spiracles remain open in normal air but in air plus CO_2 they nearly close as the abdomen contracts, and open as the abdomen expands.

3. When the bee is in normal air there is a strong tidal flow of air in and out of the first thoracic spiracles, but only a weak and variably directed stream of air through the body.

4. It is deduced that when an active bee is producing metabolic CO_2, directed streams of air enter the first thoracic spiracles and the abdominal spiracles and leave via the propodeal spiracles.

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* This behaviour of the spiracles is not likely to be dependent directly upon oxygen lack since Mr J. Simpson has shown that in an atmosphere which is 45 % CO_2 the bee becomes completely motionless, but oxygen tension must be lower than 7 % before a perceptible change in the activity of the bee can be seen.

THE NERVES AND MUSCLES OF MEDUSAE

I. CONDUCTION IN THE NERVOUS SYSTEM OF *AURELLIA AURITA* LAMARCK

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(*Received* 1 *December* 1953)

INTRODUCTION

In their classical works on *Aurellia*, both Eimer (1874) and Romanes (1876) demonstrated the propagation of the wave of contraction of the circular muscle in all directions across the bell. The rapid transmission of this contraction wave with a velocity of about 50 cm./sec. is responsible for the symmetry of the movement at each beat when the animal is swimming. Upon his discovery of 'varicose' fibres spreading out from each of the eight marginal ganglia, Eimer at once assumed that he had found nerves responsible for the propagation of the contraction wave. Romanes was more cautious; 'lest the fibres which Mr Shäfer (1878) describes as nervous should eventually prove to be the anatomical structures that are concerned in performing the physiological functions...I adopted...a neutral term' (he called the physiological pathways 'lines of discharge').

The passage of a wave of contraction along a strip of tissue cut from the bell is eventually prevented if the strip is made narrower by gradual removal of tissue. Romanes pointed out that the interruption suddenly appears at one point only, and persists at that point, in a strip that may be 1 cm. wide. Romanes was also able, in the following way 'to locate very precisely the line through which physiological continuity had been established'. While stimulation at any other part of a small area was always followed by only a small local contraction, stimulation of this 'line of discharge' produced a general contraction.

Bethe (1903) showed that a wave of contraction of the circular muscle passes muscle-free areas of the bell of *Rhizostoma*, but this tells us nothing about conduction in the regions of muscle. Mayer (1906) showed that the contraction wave passes damaged areas containing nerve fibres but is not transmitted across a previously damaged area where muscle fibres alone have regenerated. Mayer also showed that the wave passes an area where the muscle is anaesthetized with magnesium ions. These experiments leave open the question whether there is, in addition to the normal contraction wave, a slower conduction through normal muscle from fibre to fibre. Moreover, Mayer's identification of the separate elements is open to doubt. In a study of the behaviour of Scyphozoa, Bozler (1926) described reactions where decremental conduction is involved, but these are never concerned with the normal propagation of the beat. Bozler also pointed out that the contraction wave is unlikely to be propagated through the muscle alone because the velocity of the contraction wave is the same along the line of the muscle fibres as across it. In the polyp *Calliactis*, Pantin (1935) measured the refractory period of the through-conducting system, and Bullock (1943) measured that of several species of medusae. These results show that the most rapid conducting system is distinct from the muscle and they are the best physiological evidence of nerves we have, but they do not disprove conduction by additional means, and none of the experiments definitely locate the pathway of the excitation.

Evidence will now be presented to show that the excitation associated with the contraction wave is conducted in either direction at least by the thicker nerve axons and that its passage is accompanied by an electrical impulse of the usual type.

THE FUNCTION OF THE OBSERVED AXONS

A piece of the bell was mounted on a microscope stage with the subumbrella epithelium uppermost. The larger fibres of the nerve net of such a preparation could be clearly seen by use of a phase-contrast microscope or oblique illumination (Horridge, 1953). Moreover, the fibres proved to be strong enough to tolerate a certain amount of manipulation under the microscope. This made it possible to show that some of the observed fibres in the epithelium are in fact essential for the propagation of the contraction wave.

With a little care it was possible to make a narrow bridge of tissue with a single observable axon going across in a bridge of epithelium from one part of a piece of *Aurellia* to another part. Supported on wax or cork, the bridge was held motionless under the microscope by several pins to prevent damage which might result from contraction of the preparation. To give the fibres of the circular muscle a chance to conduct the excitation across the bridge, some of the bridges were cut along the line of the muscle. The bridge was examined under a microscope and gradually made narrower and narrower until only one observable axon went across. During this process of whittling down, the propagation of the excitation across the bridge was tested from time to time (Fig. 1). In many experiments the propagation broke down while the bridge still contained several axons, but one could not tell whether these had no connexion with the through-conducting system or were merely damaged by the pulling and tearing. Running across the bridge were plenty of muscle fibres which did not appear to be damaged.

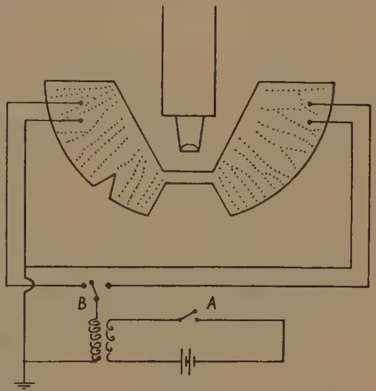

Fig. 1. Arrangement of the experiment with a single fibre in a bridge between the pieces of *Aurellia*. The switch *A* breaks the primary circuit, and the switch *B* directs the stimulus to one side or the other.

In six successful experiments a bridge was prepared with a single axon running across, and was found to conduct the contraction wave in either direction. In one experiment propagation was in only one direction and remained so after stimulation. Again it was impossible to decide whether this polarization, which other authors have found in long narrow bridges, was due to damage to the axons and their connexions. In each case section of the axon in the bridge stopped the propagation, and I have never succeeded in making a bridge which would conduct without an axon.

It is not likely that in each case failure to conduct after section of the nerve fibre was in fact due to damage of the muscle by the operation, and it is more likely that it was due to section of the nerve. A bridge containing muscle fibres but no nerve axon would not conduct and some bridges would not conduct although apparently undamaged axons were visible. In these cases narrow strips of muscle did not conduct the contraction wave and it seems unlikely that wider pieces of muscle would behave differently. This experiment showed that the contraction wave was propagated through the nerve fibres, not through the muscle, and that the excitation could be conducted in either direction along a single nerve fibre.

ELECTRICAL RECORDING FROM A NERVE FIBRE

Method

The diagram, Fig. 2, shows the arrangement of the apparatus for electrical recording. A microscope *A* with a 16 mm. objective had on its stage a flat dish made of a piece of glass 10 by 20 cm. with wax walls. A piece of *Aurellia*, lying in this dish with subumbrella epithelium uppermost, was kept moist with sea water from a hand pipette. In some cases it was necessary to flatten the piece of the bell by cutting off jelly from the exumbrella side of the thickest part of the wedge. It was found that the greater clarity obtained by the use of a phase-contrast microscope was not worth the time and trouble in making the continual adjustments as the preparation moved.

Attached to the same base as the microscope was a micromanipulator *B* which held the recording electrode in the field of view. With this a nerve fibre was picked up on a fine platinum wire and raised into the air above the surface of the epithelium.

The piece of *Aurellia* rested on an earthed plate arranged to avoid obstruction of the light passing through the microscope. All the apparatus rested upon a large sheet of galvanized iron, to which the whole was earthed. Fifty-cycle hum in the recording system could be avoided by keeping far from mains circuits, but extensive screening was necessary in a small room.

A screened lead from the electrode led to a condenser-coupled amplifier of usual design with a long time constant. The output of the amplifier was visible on a single-beam cathode ray tube *S* with a time-base, and also deflected one of the spots of the double beam tube *Y*.

The movements of a part of the *Aurellia* near the electrode were followed by the device shown at *R*. A potential difference from an h.t. battery was maintained between two electrodes in a bath of distilled water. Between these electrodes a light wire lever was suspended. To this lever was attached a piece of cotton fastened at the other end by a hook to the *Aurellia*. Parts of this cotton were soaked in Bakelite varnish and then baked. This prevented electrical leakage from the movement recorder to the amplifier. The lever was connected to a terminal by a thin silver wire, which served as a light spring. The use of distilled water for the medium of the potential gradient prevented extensive electrolysis. For a shift control of the recording spot, some point in the middle of the h.t. battery *E* was connected to earth.

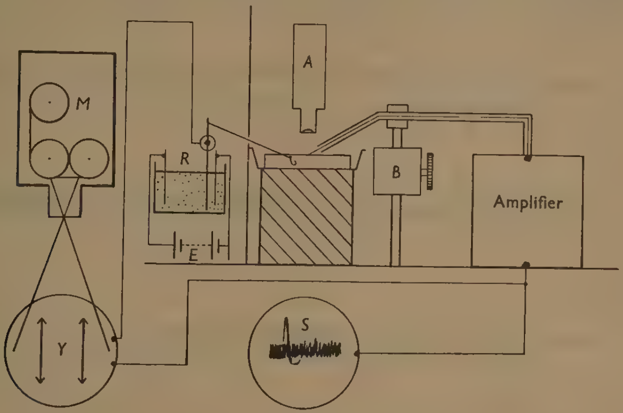

Fig. 2. Diagram of the apparatus for recording the electrical effects and the movement. Explanation in the text.

A camera *M* with sensitive paper running between two drums followed the movements of both spots on the tube *Y*. In the records (Figs. 3, 4), the upper trace represents the output of the amplifier and the lower trace represents the corresponding change of the movement recorder.

It was unavoidable that the electrical and mechanical records were from points separated by a few centimetres. According to the direction in which the wave of contraction travelled as it passed these two points, the interval between the action potential and the record of the movement could vary a little. On account of this uncertainty, the accurate measurement of the latent period between the nerve impulse and the movement was not possible. This difficulty could have been overcome by the use of artificial stimulation, but the problem of stimulus escape made this impossible.

Results

At each spontaneous beat of the bell an action potential from the nerve was recorded, together with the corresponding movement of the muscle.

Of all the fibres tested only about one in thirty gave any result. An electrical impulse was obtained from eleven different specimens; in each case it was of the same characteristic appearance shown in Fig. 3. The nerve impulse appeared consistently once at each spontaneous beat of the bell, as shown in Fig. 4, and at no other time was an electrical effect of this kind observed. In Fig. 3, the electrical impulse is shown on a background of noise and below is the corresponding response of the muscle. This figure also demonstrates the consistency of the response, which is in fact to be expected from a preparation of a single axon.

If the electrical change had its origin in the muscle fibres one would expect to find a larger proportion of active preparations and the same results would appear with the electrodes placed on the muscle. Neither of these expectations was realized. When the electrodes were placed on the surface of the muscle either nothing was observed at each contraction or there were slow electrical changes that lasted for more than a second. Occasionally a fast spike, as in Fig. 3, could be picked up from a piece of raised epithelium which contained both nerve cells and muscle fibres. Sometimes the nerve axon on the electrode showed both the spike and the slow potential change which accompanied the movement. Typical action potentials were only found with a living nerve fibre on the electrode. There seems no doubt that the single action potential at each beat came from the axons of the nerve net, and the slow changes can be attributed to summation of the effects of numerous muscle fibres.

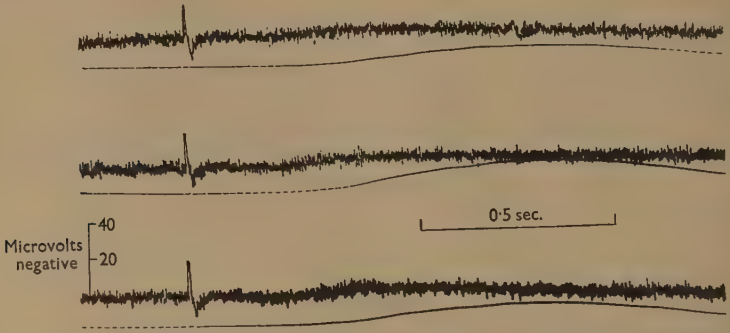

Fig. 3. Part of a typical trace showing the nerve impulse on a background of noise from the amplifier, and in the lower trace the accompanying movement.

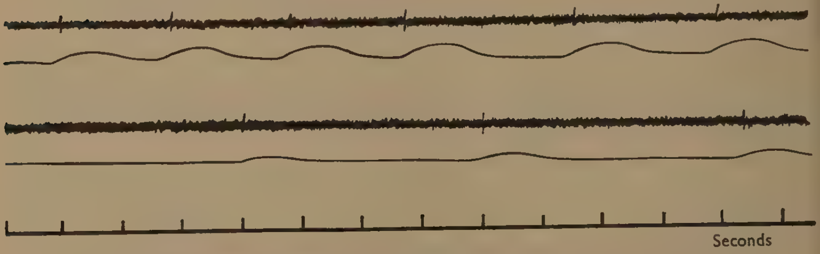

Fig. 4. The electrical and mechanical effects. The electrical response appears delayed relative to the mechanical response because recorded some distance from it.

DISCUSSION

The experiment with a nerve fibre in a bridge of tissue has shown that the fibres formerly thought to be nerves do in fact conduct the excitation which leads to a contraction of the muscle. The nerves are clearly visible with suitable illumination, and these observations suggested the possibility of recording an electrical concomitant of the nerve impulse. Such an experiment is interesting on several accounts. Pantin (1935) showed that the excitation in the through-conducting part of the nervous system of *Calliactis* has essentially the same properties as the nerve impulse of higher animals. It is of interest to know whether the expected electrical concomitant of the nerve impulse can be recorded from the nerves of a Coelenterate. In fact, an action potential has been recorded, and this provides a confirmation of earlier conclusions and shows a more complete resemblance to the nerves of higher animals.

The propagation of the contraction wave across the bell is only one of several examples of transmission of excitation across the bell of Scyphozoa. Romanes (1876) demonstrated transmission of a wave of tentacle retraction in *Aurellia* and also the initiation of a beat from a tentaculocyst following stimulation at a distant point. He distinguished this 'excitational continuity' from the 'contractional continuity' of the wave of contraction. Bozler (1926) has demonstrated in *Rhizostoma*, and also in *Pelagia*, many examples of transmission of excitation other than the contraction wave.

The observations presented here show that the large nerve fibres carry the excitation which leads to a contraction wave. This is shown by the experiment where a single nerve fibre is sufficient and necessary for conduction across a narrow bridge between two pieces of tissue. In both Actinozoa (Pantin, 1935) and Scyphozoa (Bullock, 1943) this wave of contraction is followed by a refractory period. This refractory period indicates that a single impulse has gone from axon to axon throughout the effective system, which has now been demonstrated by direct recording from a single axon at each spontaneous contraction wave. A single impulse produces a single contraction, if the muscle is in a condition to respond. A contraction is therefore produced by the leading impulse of any train of impulses, so that any excitation in the form of impulses which is propagated along the system must be accompanied by a movement of the muscle. There are the following observations:

(1) A single impulse produces a contraction.
(2) The whole system (effectively) is active at each contraction wave.
(3) The direction that the excitation takes across the bell is of no importance.
(4) More than one kind of excitation may be transmitted.

On safe grounds it is assumed that only one kind of nerve impulse can travel through each axon, and that the effect of this impulse does not vary from occasion to occasion, so that it cannot produce different effects at different times. With these limitations it does not seem possible that each of the observed nerve axons can transmit excitation of two kinds, only one of which is accompanied by a contraction wave. To use Romanes's terminology, it has now been shown that 'excitational continuity' cannot be maintained in the same conducting system as 'contractional continuity'. There must be a second conducting system, independent of the 'contractional' network, for the transmission of excitation which is not accompanied by a contraction wave. This second system is diffuse; there is evidence to suggest that it is a nerve net; altogether it seems safe to conclude that there are at least two nerve nets. The characters of the two nerve nets will be discussed more fully in a later publication.

SUMMARY

1. It has been shown that a single large bipolar nerve cell in the subumbrella epithelium of *Aurellia* is necessary and sufficient to conduct the contraction wave across a narrow bridge from one part of the bell to another.

2. This nerve impulse is accompanied by an electrical impulse of the kind usually recorded from the nerves of higher animals.

3. These nerve cells are concerned only with the excitation leading to a contraction wave. There must be other pathways for other transmitted excitation.

The material was collected on the coast of Norfolk, and the first experiments were done either at a temporary laboratory on the coast, or at Cambridge. I would like to thank Mr Chestney for his help and accommodation at Brancaster, and Dr Pantin for encouragement throughout the course of the work. The electrical recording was done at the Marine Station, Millport, where *Aurellia* was abundant in June 1952, and I should like to thank the director, Mr Ford, and his staff for all the facilities they provided.

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IONIC REGULATION IN THE PALAEMONID PRAWN *PALAEMON* (=*LEANDER*) *SERRATUS*

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(*Received* 20 *February* 1954)

I. INTRODUCTION

It has now come to be accepted that osmotic regulation of body fluids of invertebrates is secondary to ionic regulation (Pantin, 1931), and a number of papers dealing with the relation between the two in Crustacea has appeared in the last two decades (Krogh, 1939 for summary; Robertson, 1939, 1949, 1953; Webb, 1941). The greater part of this work has been concerned with animals whose natural environment is sea water, and which can tolerate dilutions of this medium. The study of osmoregulation of palaemonid prawns (Panikkar, 1941) has established this group as one which is normally hypotonic to a sea-water environment—a character which is held in common with a number of other decapods, viz. *Metapenaeus* (Panikkar & Viswanathan, 1948), *Crangon* (Broekema, 1942), grapsoid crabs (Pearse, 1932; Schwabe, 1933; Baumberger & Olmsted, 1928; Jones, 1941; Edmonds, 1935; Conklin & Krogh, 1938) and ocypodid crabs (Pearse, 1932). All of these animals show an osmotic independence of the medium, and although not all have been investigated for ionic regulation, it is to be expected from Pantin's thesis that they will all show well-developed ionic regulation. The role played by the antennal gland in maintaining this regulation has been studied in only a few examples, viz. *Eriocheir* (Scholles, 1933), *Maia* (Bialaszewicz, 1932), *Cancer* (Robertson, 1939), *Carcinus* (Nagel, 1934; Bethe, von Holst & Huf, 1935; Webb, 1941), *Cambarus* (Lienemann, 1938), *Potamobius* (Herrmann, 1931).

The palaemonid prawns are convenient for a study of this hypotonicity, since different species show varying degrees of osmotic independence. Panikkar (1941) investigated the osmotic behaviour of three species, but there appears to be no published data for the concentration of inorganic ions in the body fluids. Panikkar established the hypotonicity of the blood and urine of *Palaemon* (=*Leander*) *serratus* (Pennant), *P. elegans* (Rathke)=*Leander squilla* (L.) and *Palaemonetes varians* (Leach), when living in sea water, and he also demonstrated their hypertonicity to the medium in brackish water of varying dilutions. Of the group, *P. varians* is the most adaptable to changes in the medium, *Palaemon serratus* the least. Individual specimens of *Palaemonetes varians* can survive salinities of the order 0–5 % NaCl without much change in the internal medium, and a completely fresh-water variety of this species is common in some rivers of southern Europe (Boas, 1898).

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Osmotic behaviour of palaemonids

The hypotonicity of the blood to sea water when the animals are living in that medium was demonstrated by Panikkar (1941) by means of vapour-pressure measurements. *Palaemonetes varians* has a blood equivalent to 2·3% NaCl (Δ 1·38° C.), *Palaemon serratus* 2·6–2·8% NaCl (Δ 1·56–1·68° C.) and *P. elegans* 2·6% NaCl (Δ 1·56° C.) (calculated from the relationship Δ/0·6 = % NaCl, Ramsay, 1949). *Palaemonetes varians* is almost completely homoiosmotic at about 2% NaCl and varies only by about 0·8–1·0% NaCl for external salinities from 0 to 5%. Thus it can tolerate, with small internal changes, a range of salinity from water which is nearly fresh to 5·2% NaCl. This behaviour is less well developed in *Palaemon serratus*, which has a much more limited tolerance to varying salinity in the environment. When the environment is diluted to 2·5% NaCl (which is about isotonic with the blood) the concentration of the blood is maintained; but in greater dilutions there is a steady fall in blood concentration. *P. elegans* is very similar to *P. serratus*, but the homoiosmotic behaviour is a little better developed, so that it falls into an intermediate position between the other two species. When any one of these prawns is transferred to very dilute media, the blood osmotic pressure falls for 14–24 hr., but after this there is a slight compensating rise, and a steady state is thereafter maintained.

The urine of all three species is nearly isotonic with the blood in all conditions of external salinity. Different parts of the excretory system do not seem to have any salt- or water-regulating mechanism, as the osmotic pressure of fluids from different parts of the gland in *P. serratus* do not show any significant differences. During the moult there is a slight rise in the blood osmotic pressure; this is followed by the absorption of water and a return of the blood osmotic pressure to a normal level a few days later.

In an attempt to elucidate further the regulation of the blood in prawns, chemical analyses were made of the blood and urine of *P. serratus*. This species was chosen because it is larger than the others available and yielded quantities of fluid which it was possible to analyse by ordinary semi-microchemical methods. This species is the least homoiosmotic of the four which are easily available in Britain, so that any regulation of inorganic ions apparent in this species may be expected in greater degree in the others.

II. METHODS

Blood was collected from *P. serratus* by inserting a glass cannula into the dorsal side of the abdomen, just in front of the telson, and applying gentle suction. In this way blood was collected from the extensive sinuses of the abdomen. If insufficient blood was collected, a second cannula was inserted into the heart or pericardium from the back of the thorax and the blood gently sucked out. A rather large specimen would provide 0·2–0·3 ml. blood, but samples as small as 0·02 ml. could be used for some analyses. Urine samples were collected by a glass cannula from the 'nephroperitoneal' or 'epigastric' sac (a dorsal backwardly projecting extension of the bladder), or from the excretory opening of the gland. To collect from the sac, a small cut was made in the ridge of the rostrum and the pointed cannula pushed into the sac from an antero-dorsal position; to collect from the excretory pores, the animal was laid on its back beneath a binocular microscope, a sharply tapered cannula fitted into one of the openings of the gland, and the urine drawn up. The former procedure was less satisfactory, since samples could occasionally be contaminated with blood from one of the anterior arteries. For this reason, most of the later collections were made from the excretory pores. Volumes of 0·1–0·01 ml. were obtained.

The volume of samples, when necessary for the calculation of ion concentrations, was measured by weighing an identical quantity of distilled water in the collecting cannula. The results are thus expressed primarily as mg./ml. blood. Samples could not be weighed directly as the blood coagulates very rapidly. The concentrations can be expressed in other terms of concentration by using the experimentally determined estimates of water content and specific gravity.

The blood and urine samples were taken from both males and females, and were analysed without removal of corpuscles. Both these procedures will add to the variability of the results, since Panikkar (1941) found a difference of 0·1 % NaCl concentration in the blood of ovigerous females compared with males. The removal of corpuscles by centrifugation was found to be impracticable with such small samples, since manipulation would reduce their volume still further and so lead to increased errors in the analyses used. It was hoped that the concentration differences apparent after sojourn in different salinities would be sufficiently large to make these variations relatively insignificant.

The procedures for chemical analysis were based on those of Robertson & Webb (1939), since these are suited to the samples available. There were some modifications introduced for the rather smaller quantities of fluid analysed. These methods have been reviewed in a previous paper (Parry, 1953). Chloride determinations in this investigation were all done by Conway's microdiffusion technique (1950). In almost all the analyses samples of standard sea water (Copenhagen) were treated in exactly the same way as the blood or urine samples; the final calculations of the ion concentration could thus be made by a comparison of titration volumes, precipitate weights, etc. In this way almost all personal errors due to slightly different temperatures, or times for reaction, are minimized. In many cases the volumes of samples were also determined and could be used as a check of the accuracy of the method. The results were calculated by comparison with the ionic concentrations in sea water given by Sverdrup, Johnson & Fleming (1942, p. 173).

III. EXPERIMENTAL RESULTS

Composition of the blood of animals living in sea water

The concentrations of inorganic ions found in the blood of animals living in their normal sea-water environment (sea water from Plymouth) is shown in Table 1. The results are expressed here as mg./ml. blood, since the volume of the samples was measured at the time of the analysis. These values have been converted to m.equiv./l.; they may also be converted to m.equiv./kg. water from the estimate of the water content of the blood.

The water content of the blood was determined by drying weighed samples at 110° C. to a constant weight, and gave an estimated mean of 877 mg./ml. blood (nine observations). The original values for ion concentration as mg./ml. blood can be expressed in terms of molality by using this figure.

The specific gravity of the blood was determined by weighing a sample of blood in its collecting tube, and then weighing an identical volume of distilled water in the washed tube. The mean of nine such determinations was 1·030.

Table 1. *Concentration of some inorganic ions in the blood of* Palaemon serratus *living in sea water*

Ion	mg./ml. blood	m.equiv./l.	$\frac{\text{mg./ml. blood}}{\text{mg./ml. sea water}}$ %
Sodium	9·09 ±0·26 (15)	394	79
Potassium	0·290±0·021 (8)	7·7	70
Calcium	0·500±0·012 (9)	25·2	115
Magnesium	0·307±0·058 (13)	25·2	22
Chloride	15·20 ±0·13 (20)	430	74
Sulphate	0·948±0·173 (13)	5·2	9

Bracketed figures are the number of determinations.

The total mg. ions/kg. water analysed in blood is about 5% less than that calculated from Panikkar's (1941) measurements of the osmotic pressure:

By analysis, 856 mg. ions/l. blood ≡ 876 mg. ions/kg. water.
From O.P. (Δ1·56° C.), 914 mg. ions/l. blood ≡ 948 mg. ions/kg. water.

This may indicate that either the estimate of Δ is too high, or that the analyses give a result which is about 5% too low. It seems possible that the estimate of anions is some 20 m.equiv. too low, as there are only 436 m.equiv. anions/l. to balance 452 cations. If the anions totalled about 450, the total mg. ions/l. would be about 900. (This difference of 5% is well within the error of determinations of the concentration of individual ions. Panikkar (1941), while he gives the range of his vapour-pressure determinations, does not give any standard errors of the means.) A determination of the total base in blood (by the method of Hald, 1933, 1934) gave a value of 446 m.equiv./l. (nine observations). This compares fairly well with the sum of the cations for the individual analyses (452 m.equiv.), indicating that it is the figure for the sum of the anions which is too low. The deficit must be entirely due to a low figure for chloride, since sulphate is relatively much less important in this numerical sense. The chloride analyses in question were mostly done during June, July and August when Panikkar (1941) records that the osmotic pressure is lower than in winter.

The concentration of proteins in the blood has not been measured directly, but some estimate of their quantity may be made from the calculation of total salts and water in the blood:

Volume	Weight	
1·000	1·030	Total
0·850	0·877	Water
0·150	0·153	Solutes (by difference)
0·026	0·027	Salts (from analyses)
0·124	0·126	Proteins and unidentified salts

Proteins thus appear to make up about 12 % of the total. This seems high compared with other decapods, where it ranges from 2·25 to 7·38 % (Prosser *et al.* 1950, p. 104) and 2·9 to 8·0 % (Robertson, 1953). Among the invertebrates similar quantities of protein in the blood have been found only in some cephalopods (*Eledone*, *Loligo* and *Sepia*, Robertson, 1949, 1953). That there is a largely variable quantity of protein or other organic material present in the blood has been shown by measurements of the refractive index (using a Leitz-Jelley refractometer) of the blood of this and other species of palaemonids. The blood of *Palaemon serratus* in Plymouth sea water has an index of 1·354, while the salts in the blood only account for an index of 1·340, so that there must be a fairly high proportion of the index due to proteins or other organic solutes. Some of this high concentration of proteins may very well be due to the use of whole blood rather than plasma.

The proportions of individual ions in the blood will be discussed in a later section dealing with their concentrations in media of other salinities.

Table 2. *Concentration of some inorganic ions in the urine of* Palaemon serratus *living in sea water*

Ion	mg./ml. urine	m.equiv./l.	$\frac{\text{mg./ml. urine}}{\text{mg./ml. blood}}$ %
Sodium	7·45 ± 0·38 (13)	324	82
Potassium	0·250 ± 0·023 (9)	6·6	86
Calcium	0·476 ± 0·025 (9)	24·0	95
Magnesium	2·060 ± 0·270 (12)	168·6	670
Chloride	16·20 ± 0·04 (26)	458	106
Sulphate	0·948 ± 0·173 (13)	19·8	380

Bracketed figures are the number of determinations.

Composition of urine of animals in sea water

The analyses for urine, like those for blood, do not completely correspond with data derived from Panikkar's (1941) measurements of the osmotic pressure (Table 2). The osmotic pressure of urine is the same as that for blood, the mean value of Δ being 1·56° C., corresponding to 948 mg. ions/kg. water $\equiv$ 885 mg. ions/l. urine (using the estimate of water content of the urine). The total mg. ions/l. urine from analyses is 895. There is some excess of cations compared with anions (*c.* 40 m.equiv.), although in view of the large concentration of magnesium present, the electro-neutrality may be complicated by the existence of non-ionized complexes.

The water content of the urine (determined as for blood) is 933 mg. water/ml. blood (nine determinations).

The specific gravity, determined in the way described for blood, is 0·977 (nine determinations). This appears surprisingly low. Determinations of the specific gravity of the urine were variable, but consistently less than unity. Nine samples were used. If the ammonium ion is present as an excretory product, this could account for a reduction in the specific gravity, if suitably large quantities were present. Of the total constituents of urine, there are 912 mg./g. water, which leaves 65 mg. for all solutes. Analysis of the inorganic ions, sodium, potassium, calcium, magnesium, chloride and sulphate, account for 27 mg. of this, so that about 38 mg. remain for other ions and excretory products. If all this 38 mg. were attributed to the ammonium ion, there would be a concentration of *c*. 4% ammonia. With this quantity of ammonia present in pure water, the specific gravity of such a solution would be 0·983. The concentration of ammonia is likely to be a variable quantity depending upon the amount of food eaten and the rate of urine flow.

The concentrations of individual ions will be discussed below.

Composition of blood and urine of animals living in hypo- and hypertonic media

Animals were kept in the laboratory in certain dilutions and concentrations of sea water in order to accentuate the differences between the internal and external environments. The media chosen were 50 and 120% of Plymouth sea water. The first was made simply by diluting Plymouth sea water with the same quantity of distilled water; the second by concentrating Plymouth sea water to twice its usual concentration, correcting this as to pH by adding drops of sodium bicarbonate, and diluting with the appropriate quantity of ordinary sea water. In this way the balance of ions should have been fairly similar to that in normal sea water, although some calcium appears to come out of solution with the initial evaporation. This sediment was kept in contact with the solution, and a large part of it appeared to dissolve again when the final medium of 120% sea water was prepared. In both these media the animals were able to survive indefinitely at 18–20° C. Experimental animals were first acclimatized for about 1 week in an intermediate salinity, and then left for at least a further week in the final salinity.

The analyses were made in exactly the same way as for animals living in ordinary sea water. The results are shown in Table 3 for blood and Table 4 for urine. The values for blood and urine of animals in sea water are added for comparison.

The correspondence of the observed mg. ions/l. blood and urine and the values of Δ for blood and urine in all three salinities is shown in Table 5. Although the calculated and observed mg. ions/kg. water are not identical, the discrepancies are not unduly large in relation to the general variability of the individual analyses, except in the case of urine of animals living in 120% sea water. In this medium the urine contains such large quantities of magnesium and sulphate that the activity of the inorganic salts in solution may well be reduced, leading to a consequent reduction in Δ. (The freezing-point depression of a magnesium sulphate solution is only 58·5 % of an isomolar sodium chloride solution.)*

The control of individual ions

The relative proportions of ions in the blood and in the medium in which the animals are kept indicate the conservation, excretion or exclusion of those ions.

Table 3. *Comparison of the composition of the blood of* Palaemon serratus *living in 50, 100 and 120 % sea water*

(Concentrations expressed as m.equiv./l.)

Medium as % sea water	Sodium	Potassium	Calcium	Magnesium	Chloride	Sulphate
50	257 ± 18	6·7 ± 0·9	28·3 ± 1·4	22·2 ± 3·8	300 ± 17	2·3 ± 0·8
100	394 ± 12	7·7 ± 0·6	25·2 ± 0·6	25·2 ± 4·8	430 ± 4	5·2 ± 1·0
120	473 ± 8	10·2 ± 1·3	29·9 ± 1·8	41·2 ± 3·4	494 ± 39	6·4 ± 1·2

Table 4. *Comparison of the composition of the urine of* Palaemon serratus *living in 50, 100 and 120 % sea water*

(Concentrations expressed as m.equiv./l.)

Medium as % sea water	Sodium	Potassium	Calcium	Magnesium	Chloride	Sulphate
50	206 ± 18	7·4 ± 0·9	25·0 ± 1·3	67·1 ± 12·6	330 ± 1·1	3·6 ± 1·0
100	324 ± 16	6·6 ± 0·6	24·0 ± 1·3	168·6 ± 22·1	458 ± 2·5	19·8 ± 3·6
120	432 ± 11	8·5 ± 1·5	29·3 ± 1·8	184·5 ± 22·2	622 ± 2·5	32·0 ± 9·3

Table 5. *Calculated and observed values of mg. ions/kg. water in blood and urine of* Palaemon serratus

Medium as % sea water	Sample	Δ° C. (Panikkar)	mg. ions/kg. (from Δ° C.)*	mg. ions/l. observed	mg. ions/kg. (from sp.gr.)†	Difference as %
50	Blood	1·03	623	590·1	608	2·4
	Urine	1·03	623	591·3	580	6·9
100	Blood	1·56	944	855·5	882	6·6
	Urine	1·56	944	918·9	901	4·5
120	Blood	1·72‡	1015	1016·0	1046	3·0
	Urine	1·72	1015	1185·4	1160	14·3

* Calculated from data in Sverdrup *et al.* (1942, p. 67).

† Calculated from observed mg. ions/l. sample, assuming that specific gravity of blood is 1·03 and specific gravity of urine is 0·98.

‡ By extrapolation of Panikkar's graph (1941).

Since the analyses in this investigation were made on whole blood and not on a dialysed plasma, a part of the differences observed in ion concentration can be attributed to the contents of the corpuscles, a part to the effects of a Donnan equilibrium, and a part to a binding of ions to the proteins present in the plasma. This last factor is likely to be of importance only with regard to the level of calcium in the blood. The differences in ion concentration in *Palaemon serratus* are for the most part considerably greater than one would expect from the Donnan equilibrium, even when the large standard error of the figures is taken into account. It would appear that the ionic differences between the blood of the animal and the medium are significant ones, and that they must be maintained by the animal in some way which cannot be attributed merely to the presence of proteins in the blood.

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* I am indebted to Mr P. C. Croghan for this information (personal communication).

In animals living in Plymouth sea water, the sodium, potassium and chloride of the blood are all about 70–80% of their external concentrations. This might be expected for sodium and chloride, since the total blood concentration is about 80% of the medium (Δ blood = 1·56° C.; Δ sea water = 1·98° C.). In many marine invertebrates which have been examined (Robertson, 1953; Prosser *et al.* 1950) the blood potassium is higher than in the medium; but this does not seem to be the case in *P. serratus*. The figure for potassium concentration has a high standard error (*c.* 7%), but even when this is considered, the potassium concentration is comparatively low.

Of the other ions calcium shows some evidence of conservation, although in the absence of any data about the binding of this ion to proteins in the blood it is difficult to estimate the level of ionic calcium. A calcium content of 115–120% of the medium could be maintained by the effect of the Donnan equilibrium and calcium-protein complexes (Robertson, 1949).

In sea water the concentration of magnesium and sulphate in the blood is remarkably low. The analyses of urine from animals in this medium show that the concentration of these two ions is very much greater than it is in the blood, while that of other ions is not so markedly different.

The relative concentrations of ions in the medium, blood and urine in all three experimental media are shown in Fig. 1. The ion concentration of the medium is calculated from data in Sverdrup *et al.* (1942, p. 173).

Sodium is at a constantly lower level in the blood than in the medium, except in 50% sea water, where it is very similar in concentration. While the osmotic pressures of blood and medium are similar in 80% sea water, the sodium concentrations are similar at a greater dilution of the medium. The sodium concentration of the urine is consistently less than that of the blood and maintains about the same proportion except at the highest salinity where the curve becomes a little steeper, and the concentrations in blood and urine approximate more closely. Perhaps this might be interpreted as an attempt to excrete a larger quantity of sodium in response to the increased salinity of the environment, although the magnitude of the change is not very great. The urine, although it maintains a constantly lower level of sodium than the blood, can hardly be considered as the main source of the control of sodium shown by the animal.

The chloride concentration in the three fluids shows a similar pattern. In the blood, chloride is constantly lower than in the medium, except at the lowest salinity where it approximates to that of the medium. In contrast to sodium, however, the urine concentration is consistently higher than that of the blood, although this difference is scarcely sufficient to account for the control of chloride, even in the highest salinity, where the difference between blood and urine concentrations is at its greatest. It appears from the graph that there is a marked increase in the excretion of chloride in 120% sea water.

The concentrations of potassium and calcium in the blood and urine show relatively only small changes with changes in the external salinity. The standard error is large in relation to the observed differences, so that the changes cannot be regarded as very significant. For potassium, the blood concentration is lower than that of the medium, except in 50% sea water, when it is rather higher in the blood, the isotonic point being at about 60% sea water. It is kept relatively constant between 50 and 100% sea water but tends to rise a little in 120% sea water. The urine concentration follows a similar curve, which for the most part lies below that for blood. In general, the blood and urine concentrations are very similar, so that the antennal gland cannot be very important in regulating potassium.

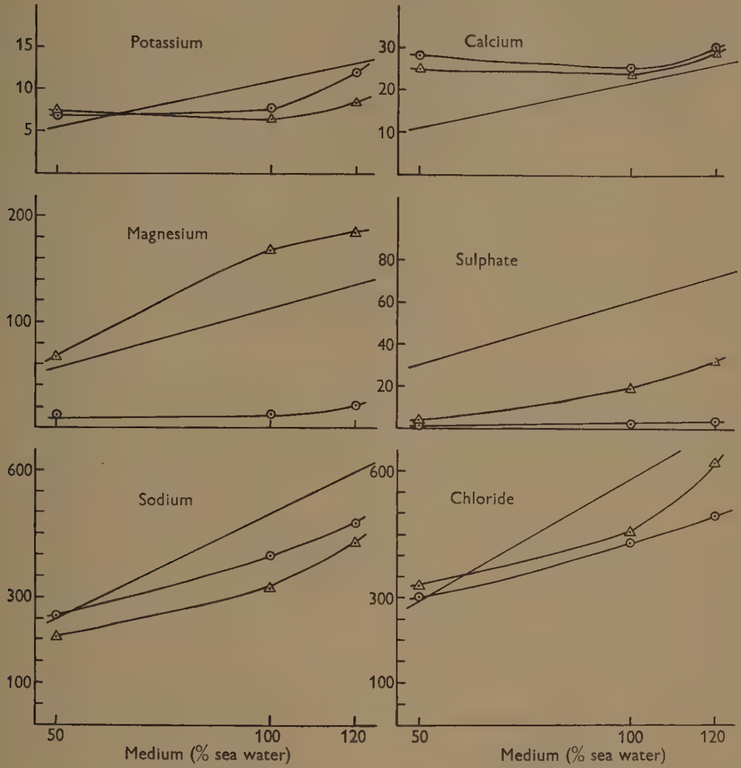

Fig. 1. Relative concentrations of ions in sea water (——), blood (⊙—⊙) and urine (△—△). Abscissae: concentration of medium as a percentage of Plymouth sea water. Ordinates: concentration of ions expressed as m.equiv./l.

Calcium is also relatively constant in blood and urine in spite of changes in the external concentration. The slightly lower level in the urine is scarcely significant in relation to the standard errors and is certainly not sufficient to account for the regulation of the ion.

The concentration of magnesium and sulphate in blood and urine with different concentrations of the medium are maintained always at very different levels. Magnesium in the blood is remarkably low and relatively constant in all three salinities. It is obvious from the high urine concentrations that this efficient regulation of the blood magnesium could be brought about by its excretion in the urine. The urine concentration rises steeply as that of the outside medium rises, although less steeply as 120% sea water is approached. In the same conditions the blood concentration rises a little as though the maximum possible rate of excretion of the ion was being approached. Sulphate in the blood is similarly very low. In 50% sea water the urine concentration is also low but rises steeply as the external concentration increases. The low excretion in 50% sea water where the blood level is still much below that outside, may indicate that sulphate is excluded from the body fluids in low external concentrations. This exclusion appears inadequate at higher concentrations, so that the antennal gland plays an increasingly important role.

IV. DISCUSSION

The principal ions of *Palaemon serratus* blood are much less concentrated than in other decapod crustaceans for which data are available. Following the lower osmotic pressure of the blood of animals in sea water, the concentrations of sodium, potassium and chloride are all 70–80% of the outside concentration. Calcium, on the other hand, is maintained at a level slightly higher than that of the medium, perhaps indicating the degree of binding to proteins in the blood. Conversely, magnesium and sulphate are proportionately much lower than in other marine decapods. This could be an adaptation to an estuarine invasion, especially in the case of sulphate, since an efficient means of regulating this ion is of relatively greater importance in waters which may possibly contain a greater proportion than sea water (Howes, 1937). The low value of the ratio of magnesium to other ions in the blood places this prawn with the most active crustaceans (Robertson, 1953). Slow-moving forms such as spider crabs have a ratio (Na + K/Ca + Mg) about 80–100% of that of sea water; most active forms have a value less than 50% that of sea water. *P. serratus* may be compared in this respect with *Homarus* and *Nephrops*, with which it has indeed many similarities of behaviour and activity.

Without some indication of the quantity of urine produced in different circumstances it is not possible to deduce completely the function of the antennal gland. The volume of urine produced in different salinities has been measured in a related palaemonid species and will be the subject of a further paper. While the inorganic analyses of urine do not necessarily demonstrate the importance of excretion in maintaining the osmotic control of the animal, they do indicate how it maintains ionic control, and make possible some deductions as to the mechanism of urine production.

The analyses of sodium, chloride, potassium and calcium in urine are, on the whole, very similar to those of blood, and their concentrations would not be incompatible with the conception of the urine as an ultrafiltrate of the blood. The much higher concentrations of magnesium and sulphate in urine could result from some active excretion of these ions after the formation of an ultrafiltrate. The lower concentration of sodium, and the higher concentration of chloride in the final fluid produced, might result from a necessity to balance the fluid ionically after the influx of magnesium and sulphate (and the ammonia which is presumed to be present). The comparatively constant proportion of sodium and chloride in blood and urine, independent of the external salinity, suggests that the ultrafiltration is unaffected (except perhaps in its rate) by the external conditions or the internal conditions resulting therefrom.

By analogy with the vertebrate glomerulus we can imagine that the ultrafiltrate is produced in the end-sac of the antennal gland, and that the excretion of additional magnesium, sulphate and perhaps ammonia into this ultrafiltrate takes place along the length of the nephridial canal, together with the reabsorption or secretion of small quantities of other ions in order to preserve electro-neutrality. The antennal glands of palaemonid prawns lack any segment such as the tubule of fresh-water crayfishes which might perform a salt-absorbing or water-regulating function, and there is certainly no experimental evidence that this takes place. The bladder and epigastric sac do not appear to play any part in the chemical elaboration of the urine. The histology of the gland (Grobben, 1880; Weldon, 1889; Marchal, 1892; Allen, 1892; Cuénot, 1895; Patwardhan, 1937) and its blood supply are in accord with this conception of the function of the antennal gland.

Some part of the ionic regulation of the animal can thus be said to be performed by the antennal glands, but the analyses do not tell us how the osmotic regulation is brought about. Presumably this must be effected extra-renally.

V. SUMMARY

1. Analyses have been made of the blood and urine of *Palaemon serratus* for the inorganic ions Na, K, Ca, Mg, Cl, SO_4, the animals being kept in 50, 100 and 120 % sea water.

2. When the animal is in 100 % sea water the concentrations of ions in the blood, expressed as percentages of their concentrations in the medium (to the nearest 5 %) are as follows: Na, K and Cl, 85 %; Ca, 105 %; Mg, 20 %; SO_4, 10 %.

3. When the animal is in 50 % sea water the corresponding figures are: Na and Cl, 105 %; K, 120 %; Ca, 200 %; Mg, 20 %; SO_4, 10 %.

4. When the animal is in 120 % sea water the corresponding figures are: Na, K and Cl, 85 %; Ca, 115 %; Mg, 30 %; SO_4, 20 %.

5. The concentrations of Na, K and Ca in the urine are always slightly ($\leqslant 20$ %) less than their concentrations in the blood. The concentration of Cl is slightly greater in the urine than in the blood (10–20 %) and the concentrations of Mg and SO_4 are very much greater, by factors of up to 7 times. The relative concentrations

of ions in blood and urine do not change substantially with changes in the external medium.

6. The antennal gland, although it plays no part in purely osmotic regulation, is no doubt partly responsible for maintaining the low blood concentrations of Mg and SO_4.

I should like to thank Prof. Sir James Gray and Prof. H. Munro Fox for the hospitality of their departments during the course of this work. My acknowledgements are also due to Dr J. A. Ramsay and Dr J. D. Robertson for advice and criticism, and to Prof. H. Munro Fox for reading the manuscript. For a part of the time engaged on this research I was in receipt of a Science Scholarship from the University of New Zealand, and latterly of a research grant from the Zoology Department of Cambridge, for both of which I should like to record my appreciation.

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THE RATE OF URINE PRODUCTION OF *ANODONTA CYGNEA*

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(*Received* 26 *January* 1954)

INTRODUCTION

It is difficult to measure directly the rate of urine production in the fresh-water lamellibranch *Anodonta cygnea* because the excretory pore is fragile and in an inaccessible position. Picken (1937) measured the rate of filtration from the heart into the pericardium by opening the pericardium from the dorsal side. Under these conditions he found a mean rate of filtration, in ten animals, of 237 ml./day, at 17° C. The average weight of the animals, without the shell, was 50 g.

This paper reports the results of the indirect measurements of the filtration rate and the rate of urine production in intact animals. The rate of filtration was measured by the inulin clearance. The rate of urine production was inferred from the initial rate of loss of weight of animals placed in diluted sea water isotonic with the blood.

MEASUREMENT OF FILTRATION RATE

All the animals used in the experiments weighed about 100 g., with the shell. Inulin concentrations were measured by the colorimetric method of Roe, Epstein & Goldstein (1949).

Preliminary determinations were made of the inulin space of *Anodonta*. One ml. of distilled water containing 30 mg. of inulin was injected into the foot. After 4 hr. the animal was drained for 5 min., dried and weighed. The animal was then opened in a clean glass dish and two blood samples were taken from the heart. The total inulin content of the animal and the inulin concentration in the blood were then measured. The shell was dried and weighed so that the inulin space could be expressed in terms of the wet weight of the animal without the shell. The mean inulin space of four animals was 55 % of the wet weight without the shell. This value will include the contents of the pericardium and the excretory organ so that the blood content will be slightly less.

The inulin clearance rate was measured as follows. One ml. of distilled water, containing 30 mg. of inulin, was injected into the foot. The animal was then left for at least 6 hr. in clean water to allow the inulin to become uniformly distributed about the haemocoele and to allow the pericardium and excretory organs to be flushed out with the inulin filtrate. The animal was then washed and transferred to 500 ml. or 1 l. of clean water. After 4–12 hr., when the concentration of inulin in the water had risen to a convenient level, the animal was removed, dried and weighed, and the concentrations of inulin in the blood and in the water were measured.

The following conventions will be used:

b_0 = initial concentration of inulin in the blood in mg./ml.
b = concentration of inulin in the blood after t hours, in mg./ml.
c = concentration of inulin in the water after t hours, in mg./ml.
C = volume of water in ml.
B = volume of blood, as measured by the inulin space, in ml.
v = volume of filtrate produced per hour, in ml.

If it is assumed that b does not fall during the experiment then

$$cC = bvt. \tag{1}$$

A more accurate value of v can be obtained if allowance is made for the fall in b during the experiment.

After the first hour $$b_1 = b_0\left(1 - \frac{v}{B}\right).$$

After the second hour $$b_2 = b_1\left(1 - \frac{v}{B}\right)$$
$$= b_0\left(1 - \frac{v}{B}\right)^2.$$

After t hours $$b = b_0\left(1 - \frac{v}{B}\right)^t. \tag{2}$$

As the amount of inulin in the system is constant

$$cC + bB = b_0 B. \tag{3}$$

As c, B, b and B are known, b_0 and v can be calculated from (2) and (3). As long as vt is small compared with B a large error in B has only a small effect on v, and the value of v calculated is similar to that obtained from (1).

The results of seven experiments, at four different temperatures, are given in Table 1. The inulin clearance rate at 18° C. for a 100 g. animal is about 1 ml./hr., and from the difference in rate between 0 and 18° C. the Q_{10} value appears to lie between 2·0 and 2·5.

MEASUREMENT OF URINE PRODUCTION

If an *Anodonta* is placed in a solution isotonic with the blood the osmotic inflow of water through the surface should be temporarily eliminated but the production of urine may be expected to continue for a short time.

The freezing-point depressions of the blood of twenty *Anodonta* were measured by Ramsay's method (Ramsay, 1949). The mean freezing-point depression was 0·0785° C. 4% sea water, freezing-point depression 0·080° C., was used as the isotonic solution.

It is possible to obtain fairly consistent weighings of *Anodonta* when the conditions of weighing are standardized. The shells of the animals were wedged open by short lengths of glass tubing. Before weighing the animals were drained for exactly 1 min. in an upright position, and the shells were wiped dry. Weighings were made to the nearest 0·01 g. Under these conditions the standard deviation of nine successive weighings of an 80 g. animal, in tap water, was 0·15 g. The experiment was performed as follows. Animals which had previously been kept in tap water were placed in the isotonic solution and weighed every 15 min. for 90 min. The results were plotted graphically, and the rate of loss of weight obtained from a straight line drawn through the seven points. Extended experiments showed that the rate of loss of weight started to fall after about 90 min.

Table 1. *Inulin clearance of* Anodonta cygnea

Temperature (° C.)	Total weight of animal (g.)	Inulin clearance (ml./hr.)
0	94	0·26
0	101	0·23
10	109	0·30
16	124	0·44
18	134	1·05
18	124	1·20
18	113	1·30

Table 2. *Change of weight of* Anodonta *in an isotonic solution*

Time in min. ...	0	15	30	45	60	75	90
Weight in g., animal I	95·64	95·44	95·38	94·71	94·96	94·35	93·73
Weight in g., animal II	66·57	65·99	65·24	64·57	64·38	64·15	63·70

Table 3. Anodonta cygnea. *Rate of loss of weight in isotonic sea water*, 15° C.

Body weight (g.)	Loss in first hour (g.)
96	0·95
66	2·19
65	0·72
59	0·16
53	0·88
97	1·71
90	1·99
67	2·43
Mean 74 g.	Mean 1·38 g./hr. S.D. ± 0·8

RESULTS

The details of the first two experiments are given in Table 2, and the final results of eight experiments are summarized in Table 3. The consistency of the results is not good; nevertheless, they give a figure of 1·9 ± 1·1 ml./hr. for the urine production of a 100 g. animal, which is of the same order as the figure for inulin clearance.

DISCUSSION

The results of the inulin clearance experiments show that the rate of filtration in the intact animal is about 1 ml./100 g. total weight/hr., at 18° C. This is much smaller than in animals in which the pericardium has been opened (Picken, 1937). The difference may be due to a slight pressure inside the intact pericardium. The rate of filtration varies with temperature with a Q_{10} of about 2. The mean rate of urine production inferred from the loss of weight experiments at 15° C. is 1·9g./100g. total weight/hr., but the spread of the results is rather wide. Florkin (1938, Table 3, Exp. 6) found that at 17° C. a 120 g. *Anodonta* lost 0·8 g. in the first hour, in an isotonic solution. The experimental errors in the loss of weight experiments are greater than in the inulin clearance experiments, but the results show that the rate of urine production is of the same order as the filtration rate.

One of the objects of this investigation was to obtain some estimate of the rate of urine production of *Anodonta* for purposes of certain theoretical investigations which are described elsewhere (Potts, 1955).

SUMMARY

1. The inulin clearance rate of a 100 g. *Anodonta* varies from about 0·2 ml./hr. at 0° C. to 1·0 ml./hr. at 18° C.

2. The rate of urine production, inferred from the initial rate of loss of weight in an isotonic solution, is 1·9 ± 1·1 ml./hr. at 15° C., and is thus of the same order as the inulin clearance rate.

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THE ENERGETICS OF OSMOTIC REGULATION IN BRACKISH- AND FRESH-WATER ANIMALS

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INTRODUCTION

The classical work of Griffiths (1891) and Fredericq (1901) more than half a century ago showed that fresh-water animals are hypertonic to the media in which they live. If the body wall of a fresh-water animal is permeable to water the animal will suffer from an osmotic inflow of water. Overton (1904) first suggested that the excretory organ of a fresh-water animal helped to balance this inflow by producing hypotonic urine. Since this time numerous papers have been published on osmotic regulation confirming and elaborating these ideas. In particular, the work of Krogh (1937, 1938) on the active uptake of ions from dilute solutions has helped to complete a dynamic picture of water and salt balance in fresh- and brackish-water animals.

This dynamic balance can only be maintained by the continuous expenditure of energy. A number of workers have attempted to demonstrate significant variations in the metabolism of brackish- and fresh-water animals in response to alterations of the concentrations of the external media (Schwabe, 1933; Peters, 1935). Although large variations were found Peters demonstrated that much of the variation was due to changes in the tissue metabolism as the blood concentration varied. The total mass of the salt-absorbing tissues is generally so small that changes in their metabolism are largely masked by the metabolism of the rest of the tissues.

In spite of this the calculation of the minimum thermodynamic work performed in osmotic regulation is of considerable interest, as it makes it possible to examine quantitatively the effect of different variables on the minimum energy requirements, and provides a convenient basis for the correlation of a large amount of data.

Qualitative considerations suggest that the energy needed for osmotic regulation will be a function of the animal's permeability, surface area, blood and urine concentrations, and the concentration of the medium in which it is living. If a few simple assumptions are made it is possible to express the total osmotic work in terms of these variables.

THEORETICAL MODEL

In constructing the model it will be supposed that the surface of the animal is permeable to water but impermeable to salts so that extra-renal salt loss is negligible. When the animal is in a hypotonic medium water will enter by osmosis and an equivalent volume must be removed as urine if the animal is to maintain a constant volume. When urine is first formed, in the amphibians, crustaceans and molluscs, it is similar to the blood in the concentrations of solutes but has a much lower colloidal osmotic pressure (Richards, 1934; Picken, 1936, 1937). Although some work must be done in overcoming the colloidal osmotic pressure of the blood, it will be very small compared to the work which must be performed in relation to the solutes and so will be neglected in the following discussion.

The following conventions will be used:

B = concentration of blood in moles/litre.
U = concentration of urine in moles/litre.
M = concentration of medium in moles/litre.
V = volume of urine produced in litres/hour.
W = work in cal./hour.
T = absolute temperature.
R = universal gas constant, 1·986 cal./mole.

Concentrations will be measured in terms of an isotonic solution of a non-electrolyte. Strictly speaking, osmotic work is a function of the activities of the ions, rather than of their concentrations, but the error introduced by using concentrations is small.

An animal which is producing V l. of urine/hr. of a concentration U moles/l. must be losing VU moles of solutes/hr. If the blood concentration is to be maintained this loss must be replaced by absorption of solutes from the medium. The work required to transfer 1 mole of solute from a concentration M to a concentration B is

$$W = RT \ln \frac{B}{M} \text{ cal.}$$

Hence the minimum work which must be performed by the animal is

$$W = RTVU \ln \frac{B}{M} \text{ cal./hr.}$$

Many animals, e.g. *Eriocheir* and *Palaemonetes*, produce urine isotonic with the blood, but when B is greater than M less energy is required if some solutes are resorbed from the urine before it is passed to the exterior. In this case the osmoregulatory work will be performed in two parts:

(*a*) in absorbing solutes from the medium,
(*b*) in absorbing solutes from the urine.

The minimum work which must be performed to produce a hypotonic urine may be evaluated as follows.

Consider V l. of urine with a concentration of x moles/l. If solutes are reabsorbed into the blood from the urine the minimum work required to reduce the concentration of the urine from x to $x - \delta x$ moles/l. is

$$\delta W = -RTV \ln \frac{B}{x} \, \delta x \text{ cal.}$$

The work needed to change the concentration of the urine from B to U is then

$$\begin{aligned} W &= -RTV\int_B^U \ln\frac{B}{x}\,dx \text{ cal.} \\ &= -RTV\left[U\ln\frac{B}{U}+U-B\right] \text{ cal.} \\ &= RTVU\left[\frac{B-U}{U}-\ln\frac{B}{U}\right] \text{ cal.} \end{aligned} \tag{1}$$

This is the minimum work which must be performed by the excretory organ per hour. An analogous formula, $RTV\left[U\ln\frac{U}{B}-U+B\right]$, for the production of a hypertonic urine, was derived by Galeotti (1902).

As long as U is finite some solutes will still be lost and work

$$W=RTVU\ln\frac{B}{M} \text{ cal./hr.} \tag{2}$$

must be performed at the animal's surface to maintain the blood concentration. Therefore the total work performed by the animal is

$$\begin{aligned} W &= RTVU\ln\frac{B}{M}+RTVU\left[\frac{B-U}{U}-\ln\frac{B}{U}\right] \\ &= RTVU\left[\ln\frac{B}{M}+\frac{B-U}{U}-\ln\frac{B}{U}\right] \\ &= RTV\left[U\ln\frac{U}{M}+B-U\right] \text{ cal./hr.} \end{aligned} \tag{3}$$

If the permeability to water of a part of the animal's surface, area A_1 cm.^{2} is P_1 l./cm.^{2}/molar concentration difference/hr.

$$V=PA[B-M], \tag{4}$$

where $PA=P_1A_1+P_2A_2+P_3A_3 \ldots$ etc.,

for the total surface of the animal. Substituting for V in equation (3),

$$W=RTPA(B-M)\left[U\ln\frac{U}{M}+B-U\right] \text{ cal./hr.} \tag{5}$$

For most fresh-water animals M is very small compared with B, so equation (5) reduces to

$$W=RTPAB\left[U\ln\frac{U}{M}+B-U\right] \text{ cal./hr.}$$

The most efficient and least efficient values for U are $U=M$ and $U=B$ respectively. In these cases equation (5) reduces to

(*a*) When $U=M$,

$$W=RTPA(B-M)^2. \tag{6}$$

In fresh water M is negligible and

$$W = RTPAB^2. \tag{7}$$

(*b*) When $U = B$,

$$W = RTPA(B-M)\left[B \ln \frac{B}{M}\right]. \tag{8}$$

In fresh water

$$W = RTPAB^2 \ln \frac{B}{M}. \tag{9}$$

From (7) and (9) it can be seen that the production of a hypotonic urine by a fresh-water animal can reduce osmotic work by $1/\ln (B/M)$.

So far it has been assumed that the blood, urine and medium all have the same relative composition. This is never strictly true. If $U_{\mathrm{Na}}/M_{\mathrm{Na}}$ are the concentrations of sodium ions in the urine and medium respectively, then, for all constituents, equation (5) becomes

$$W = RTPA(B-M)\left[U_{\mathrm{Na}} \ln \frac{U_{\mathrm{Na}}}{M_{\mathrm{Na}}} + U_{\mathrm{K}} \ln \frac{U_{\mathrm{K}}}{M_{\mathrm{K}}} + U_{\mathrm{Cl}} \ln \frac{U_{\mathrm{Cl}}}{M_{\mathrm{Cl}}} + \ldots B - U\right] \text{ cal./hr.} \tag{10}$$

In practice W calculated from equation (10) is almost identical with W calculated from equation (5) in all brackish-water animals and only differs slightly in most fresh-water animals.

Equation (10) demonstrates a curious paradox. W is independent of the concentration of any particular constituent of the blood. Many substances occur in appreciable concentrations in body fluids although they are absent from the environment. Provided that they are effectively resorbed from the urine their occurrence in the blood has no effect on W, except as they increase the total osmotic pressure of the blood.

The effect on W of changes in B, U and M can be illustrated graphically. Four conditions will be considered:

(1) B is maintained constant at the value $B_x = 100\%$ sea water (Fig. 1, X) and the animal produces urine isotonic with the blood ($U = B_x$). Equation (5) reduces to

$$W = K(B_x - M)\, B_x \ln \frac{B_x}{M} \quad \text{(curve } X'\text{, Fig. 2).}$$

(2) B is maintained constant as in (1) and the animal produces urine isotonic with the medium ($U = M$). Equation (5) reduces to

$$W = K(B_x - M)^2 \quad \text{(curve } X''\text{, Fig. 2).}$$

(3) B decreases with decreasing M (Fig. 1, Y), so that for every value of M there is a corresponding value B_y, and the animal produces urine isotonic with the blood ($U = B_y$). Equation (5) reduces to

$$W = K(B_y - M)\, B_y \ln \frac{B_y}{M} \quad \text{(curve } Y'\text{, Fig. 2).}$$

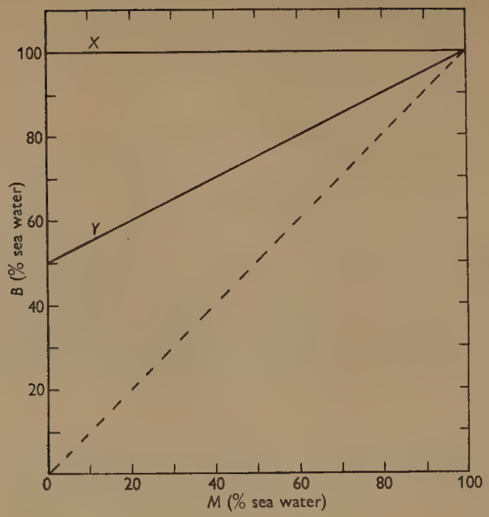

Fig. 1. Blood concentrations, *X* and *Y*, as functions of the concentration of the external medium, *M*. The broken line indicates the condition of isotonicity between blood and external medium.

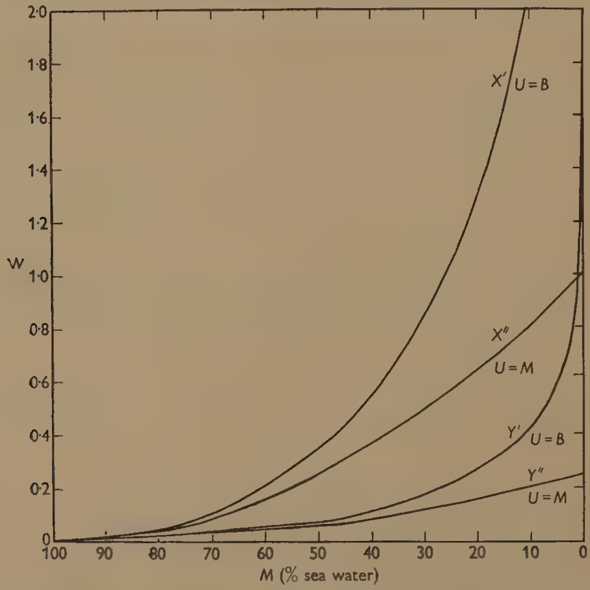

Fig. 2. Osmotic work, *W*, as functions of the concentration of the external medium, *M*. X', $B_x = 100$ % sea water (Fig. 1, *X*), $U = B_x$. X'', $B_x = 100$ % sea water (Fig. 1, *X*), $U = M$. Y', B_y decreases with decreasing *M* (Fig. 1, *Y*), $U = B_y$. Y'', B_y decreases with decreasing *M* (Fig. 1, *Y*), $U = M$.

(4) B decreases with decreasing M as in (3) and the animal produces urine isotonic with the medium ($U=M$). Equation (5) reduces to

$$W=K(B_y-M)^2 \quad \text{(curve } Y''\text{, Fig. 2).}$$

In Fig. 3 the same curves are plotted on semi-logarithmic co-ordinates to display more clearly the relations of M and W where M is very small. Hard water may be taken as 0·5 % sea water and soft water as 0·05 % sea water.

Two important points emerge from an examination of these curves:

(1) Comparing curve X' with curve Y' it can be seen that the lowering of the blood concentration affords a very substantial relief to the osmoregulatory mechanism, even when the urine is isotonic with the blood. This illustrates the effect of the relationship $W \propto B^2$, which is implicit in equation (5).

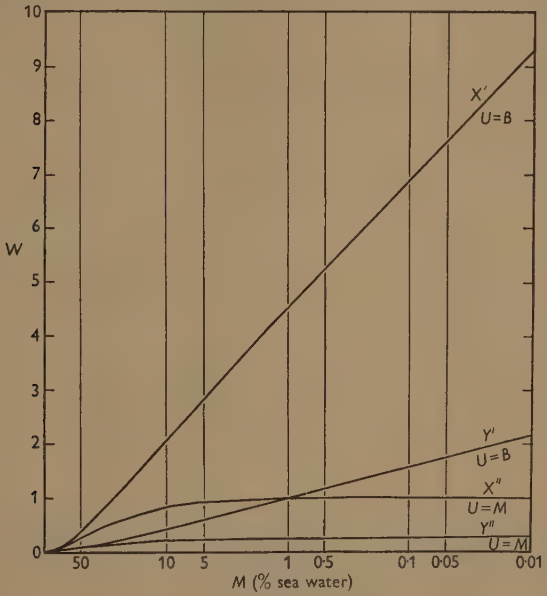

Fig. 3. Osmotic work, W, as functions of the concentration of the external medium, M. X', $B_x=100$ % sea water (Fig. 1, X), $U=B_x$. X'', $B_x=100$ % sea water (Fig. 1, X), $U=M$. Y', B_y decreases with decreasing M (Fig. 1, Y), $U=B_y$. Y'', B_y decreases with decreasing M (Fig. 1, Y), $U=M$.

(2) Comparing curve X' with curve X'' (and similarly Y' with Y'') it can be seen that the divergence between the curves, considered in relation to the amount of work, does not become important until the concentration of the medium falls below 50 % sea water. This means that the production of hypotonic urine offers little advantage to the brackish-water animal, whereas it is of enormous advantage to the fresh-water animal; it can reduce the work of osmoregulation by 80–90 %. This is of course the effect of the term $\ln U/M$ in equation (5).

Although most fresh-water animals produce urine hypotonic to the blood no fresh-water animal is known which produces urine strictly isotonic with the medium; the urine of fresh-water animals is always somewhat hypertonic to the medium. It is instructive to examine the relation between W and U in a fresh-water animal, for which purpose we will consider an animal of type Y (Fig. 1) living in soft fresh water ($M=0{\cdot}05$ % sea water). The relationship is shown in Fig. 4 and brings out a third point of general importance.

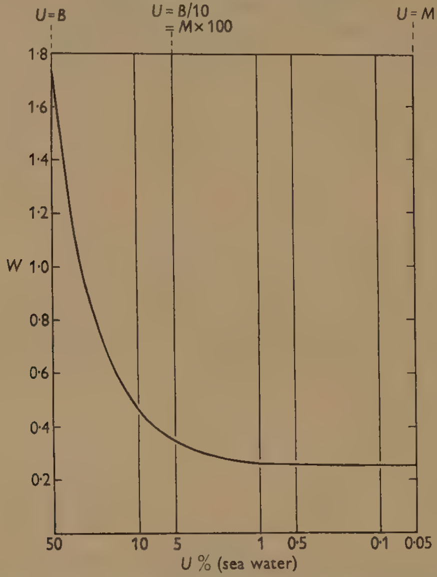

Fig. 4. Osmotic work, W, as a function of the urine concentration, U, when the blood concentration = 50 % sea water and the external medium is soft water (= 0·05 % sea water).

(3) Most of the benefit of producing a hypotonic urine is secured in the early stages of the process of reducing the urine concentration and little is gained in the later stages when the concentration of the urine approaches that of the medium. When the concentration of the urine is reduced to 1/10 of the concentration of the blood (i.e. 5 % sea water), and is still 100 times greater than the concentration of the medium (i.e. 0·05 % sea water), 93 % of the possible saving has already been achieved.

ILLUSTRATION BY EXAMPLES

The equations will be illustrated by reference to three fresh-water animals for which B, U and V have been determined, the crayfish *Potamobius fluviatilus*, the crab *Eriocheir sinensis* and the lamellibranch *Anodonta cygnea*. To facilitate comparison all calculations will be made as for 60 g. animals living in hard water ($M=0{\cdot}006$ moles/l.) at 15° C.

Potamobius

According to Herrmann (1931) *Potamobius* produces urine equivalent to 0·158 % of the body weight/hr. Picken (1936) found that in Cambridge tap water $B=0{\cdot}420$ mole/l. and $U=0{\cdot}124$ mole/l. Cambridge tap water has been anaylsed by Suckling & Porteous (1935) and contains 0·0065 mole/l. The oxygen consumption of five *Potamobius*, mean weight 46·85 g., was 47·64 mg. O_2/kg./hr. (Schwabe, 1933). This is equivalent to a metabolic liberation of energy of about 7·8 cal./animal/hr.

The following figures will be used in the calculations for a 60 g. animal:

$$M=0{\cdot}0060 \text{ mole/l.},$$
$$B=0{\cdot}420 \text{ mole/l.},$$
$$U=0{\cdot}124 \text{ mole/l.},$$
$$V=9{\cdot}5\times 10^{-5} \text{ l./hr.},$$
$$\text{Metabolic energy}=10{\cdot}0 \text{ cal./hr.}$$

From which it is calculated

$$PA=2{\cdot}307\times 10^{-4} \text{ l./hr.,} \qquad \text{by (4)}$$
$$W \text{ (total)}=0{\cdot}0367 \text{ cal./hr.,} \qquad \text{by (3)}$$
$$W \text{ (excretory organ)}=0{\cdot}0079 \text{ cal./hr.,} \qquad \text{by (1)}$$
$$W \text{ (body surface)}=0{\cdot}0288 \text{ cal./hr.,} \qquad \text{by (2)}$$
$$W \text{ (total)}=0{\cdot}3\ \% \text{ of the total metabolic liberation of energy.}$$

100 % sea water is approximately isotonic with a 1·2 molar solution of a non-electrolyte. The following calculations of W, when $M=0{\cdot}006$ mole/l., show the importance of the low blood and urine concentrations in the adaptation of *Potamobius* to fresh water.

(*a*) if $B=U=1{\cdot}2$ moles/l.

$$W=1{\cdot}31 \text{ cal./hr.;} \qquad \text{by (9)}$$

(*b*) if $B=1{\cdot}2$ moles/l., $U=M=0{\cdot}006$ mole/l.

$$W=0{\cdot}190 \text{ cal./hr.;} \qquad \text{by (7)}$$

(*c*) if $B=U=0{\cdot}420$ mole/l.

$$W=0{\cdot}0986 \text{ cal./hr.;} \qquad \text{by (9)}$$

(*d*) if $B=0{\cdot}420$ mole/l., $U=M=0{\cdot}006$ mole/l.

$$W=0{\cdot}0232 \text{ cal./hr.} \qquad \text{by (6)}$$

The value of W calculated from the measured values of B and U, 0·0367 cal./hr., is higher than the minimum possible figure, 0·0232 cal./hr., but compares very favourably with the figure calculated for a urine isotonic with the blood 0·0986 cal./hr. The urine concentration of *Potamobius* in soft water is much lower, 0·046 moles/l. (Picken, 1936), than in hard water, and the calculated work approaches the theoretical minimum even more closely.

Eriocheir

The crab *Eriocheir sinensis* is one of the few animals that can live in fresh water while producing a urine isotonic with the blood (Scholles, 1933). Scholles found that a 60 g. *Eriocheir* in fresh water had a blood concentration of 0·32 mole/l. and produced 2·5 ml. of urine/day. The oxygen consumption of *Eriocheir* in fresh water is 66·8 mg. O_2/kg./hr. (Schwabe, 1933).

For a 60 g. *Eriocheir* the following figures will be assumed:

$$M = 0{\cdot}006 \text{ mole/l.},$$
$$B = 0{\cdot}320 \text{ mole/l.},$$
$$U = 0{\cdot}320 \text{ mole/l.},$$
$$V = 1{\cdot}04 \times 10^{-4} \text{ l./hr.},$$
$$\text{Metabolic energy} = 14 \text{ cal./hr.}$$

From which it is calculated

$$PA = 3{\cdot}312 \times 10^{-4} \text{ l./hr.} \qquad \text{by (4)}$$
$$W = 0{\cdot}0757 \text{ cal./hr.} \qquad \text{by (2)}$$

This is about 0·5 % of the total metabolic energy. All this osmotic work must be done at the body surface.

(*a*) if $B = U = 1{\cdot}2$ moles/l.

$$W = 1{\cdot}69 \text{ cal./hr.}; \qquad \text{by (9)}$$

(*b*) if $B = 0{\cdot}320$ mole/l. and $U = M = 0{\cdot}006$ mole/l.

$$W = 0{\cdot}0187 \text{ cal./hr.} \qquad \text{by (6)}$$

The fall in B from 1·2 to 0·32 moles/l., on entering fresh water, reduces the osmotic work from 1·69 to 0·0757 cal./hr., but if the animal produced urine of the optimum concentration it could be cut still further to 0·0187 cal./hr.

Anodonta

According to Potts (1954) the freezing-point depression of the blood of twenty *Anodonta* was 0·0785° C., equivalent to a concentration of 0·042 mole/l. The average rate of urine production (Potts, 1954) for an animal weighing 100 g. was about 1·0 ml./hr. at 18° C. Picken (1937) found that the urine of *Anodonta* in Cambridge tap water was isotonic with 0·069 % NaCl or 0·0236 mole/l. (mean of twelve determinations).

Weinland (1918) found that *Anodonta* at 10° C. had a mean oxygen consumption of 7·38 mg. O_2/100 g./day or 0·308 mg. O_2/100 g./hr. Hiscock (1953), using the Australian species *Hyridella australis*, reported an oxygen consumption of 1·027 ml. O_2/100 g. wet tissue/hr. at 20° C. or 1·47 mg. O_2/100 g./hr. If allowance is made for the shell this would be about 1·0 mg. O_2/100 g. animal/hr. An O_2 consumption of 0·60 mg./100 g./hr. at 15° C. will be assumed.

For a 60 g. *Anodonta* at 15° C. the following figures will be used:

$$M = 0{\cdot}006 \text{ mole/l.},$$
$$B = 0{\cdot}042 \text{ mole/l.},$$
$$U = 0{\cdot}0236 \text{ mole/l.},$$
$$V = 5{\cdot}0 \times 10^{-4} \text{ l./hr.},$$
$$\text{Metabolic energy} = 1{\cdot}2 \text{ cal./hr.}$$

Hence

$$PA = 1{\cdot}42 \times 10^{-2} \text{ l./hr.},$$
$$W \text{ (total)} = 0{\cdot}0145 \text{ cal./hr.}, \quad \text{by (3)}$$
$$W \text{ (excretory organ)} = 0{\cdot}0014 \text{ cal./hr.}, \quad \text{by (1)}$$
$$W \text{ (body surface)} = 0{\cdot}0131 \text{ cal./hr.}, \quad \text{by (2)}$$
$$W \text{ (total)} = 1{\cdot}2\,\% \text{ of the total metabolic energy.}$$

Anodonta has presumably evolved from some marine ancestor whose blood was isotonic with sea water, and for purposes of the present discussion it is interesting to consider how much work it would have to do if it maintained its blood isotonic with sea water while living in fresh water of $M = 0{\cdot}006$ mole/l.

(*a*) If $B = U = 1{\cdot}2$ mole/l.

$$W = 62 \text{ cal./hr.} \quad \text{by (9)}$$

The advantage of producing hypotonic urine is illustrated by the following comparison:

(*b*) if $B = U = 0{\cdot}042$ mole/l.

$$W = 0{\cdot}0245 \text{ cal./hr.} \quad \text{by (8)}$$

(*c*) if $B = 0{\cdot}042$ mole/l. but $U = M = 0{\cdot}006$ mole/l.

$$W = 0{\cdot}0105 \text{ cal./hr.} \quad \text{by (6)}$$

From (*a*) and (*b*) it is apparent that a thirty-fold reduction in B reduces W two thousand five hundred times. The value of W calculated from the measured values of B and U, 0·0145 cal./hr., is fairly close to the minimum possible value, 0·0105 cal./hr., although U is still 60 % of B. The osmotic work performed by the excretory organ is only 10 % of the total.

W is equivalent to 1·2 % of the total metabolism. The excretory organ and the gills together account for less than 5 % of the total weight of the animal without the shell. Other possible sites of ion uptake, the epithelium of the mantle and of the foot, are difficult to estimate but probably weigh even less. Unless the metabolic activity of these tissues is very great this would imply that the efficiency of osmoregulation is fairly high, perhaps of the order of 10 or 20 %.

DISCUSSION

The equations given above define the thermodynamic minimum work which must be performed to maintain salt and water balance in a semi-permeable animal in a hypotonic medium. The real work performed in osmoregulation will be greater than this. Although little is known of the mechanisms of ion transport it is improbable that they are 100% efficient. It is by no means certain that any animal is strictly semi-permeable and some salts will be lost through the body wall. Any salt lost in this way must be replaced by increased absorption from the environment, which must increase the total osmotic work. However, experiments by Krogh (1939, p. 91) suggest that in the crayfish extra-renal chloride losses are very small. Some water may be gained by means other than osmotic uptake, e.g. by swallowing or by metabolism, in which case, although some salts may be gained from the food, the total osmotic work will probably be increased. A portion of the osmotic pressure of the urine of aquatic animals is due to bicarbonates and nitrogenous waste products. As these substances will not be replaced by absorption from the medium the osmotic work calculated from the total U will be too high. A more accurate result would be obtained by using for U that portion of the total concentration of the urine due to substances which must be replaced from the medium.

In spite of all these complications the osmotic work performed by an animal must be some comparatively simple function of these formulae which are therefore helpful in understanding the interrelationships of B, U, M and PA.

The equations help to throw light on the problem of the adaptation of marine animals to fresh water. The changes which take place in B and U, and probably PA, in fresh-water animals, are such as to reduce the strain on the osmoregulatory mechanism. It is apparent from equations (7) and (9) that the effect on W of changes in B are very much greater than qualitative considerations would suggest, and in the case of *Anodonta* it has been shown that the fall in B has reduced W several thousand times.

The reduction in W resulting from the production of a hypotonic urine depends on B and M as well as on U. In conditions where B is fairly high and M is very low, the maximum saving may be as high as 90%, but for *Anodonta* in hard water the maximum saving could only be 60% and is, in fact, less, about 40%. As M increases the value of a hypotonic urine rapidly declines, and in brackish water the production of a hypotonic offers only a small or negligible saving of work (Fig. 2). Brackish-water invertebrates almost invariably produce a urine isotonic with the blood.

The product PA is a useful concept in dealing with the problems of osmoregulation. Unfortunately, little evidence is available on the differences in PA between related marine- and fresh-water animals. However, it is clear from the comparison of *Potamobius* or *Eriocheir* with *Anodonta* that the great difference in PA between animals of similar weight is of primary importance in any consideration of the adaptation of the crustaceans and lamellibranchs to fresh water. The very high value of PA in *Anodonta*, which may reasonably be correlated with the ciliary feeding system, makes it impossible for *Anodonta* to maintain a blood concentration similar to that of a fresh-water crustacean. The energy required for such a feat would be greater than the total metabolic energy available to the animal. W can only be reduced to reasonable proportions by tolerating an exceedingly low blood concentration, thus reducing the water flux through the body. The production of a hypotonic urine makes a further valuable economy.

Beadle & Cragg (1940) pointed out that the fresh-water animals with the highest blood concentration, the crabs *Eriocheir* and *Telphusa*, produced a urine which was isotonic with the blood. They suggested that adaptation to fresh water took place in two stages. In the first stage an animal, possessed of an effective power of absorbing ions from the medium, penetrated fresh water but maintained a blood concentration of the same order as that of sea water, and produced a urine isotonic with the blood. This hypothetical stage is represented by *Telphusa* and *Eriocheir*. In the second stage the energy required for osmotic regulation was reduced by tolerating a lower blood concentration and by producing a hypotonic urine.

In view of the hypothetical calculations on *Anodonta* it is quite clear that the fresh-water lamellibranch can never have maintained a high blood concentration while living in fresh water and the two-stage hypothesis is in this case untenable. The first stage in the adaptation of the lamellibranchs to fresh water must have been the ability to tolerate a lower blood concentration, since only this could sufficiently reduce the osmotic work. This ability to withstand a lower blood concentration must depend on changes in the tissues which will be discussed in a later paper. Such an initial reduction of the blood concentration may have taken place in estuaries or brackish lakes while the animal was still poikilosmotic, but true fresh-water life would only be possible when the animal was able to maintain a blood concentration hypertonic to the environment. This would entail the power to absorb all the common ions against a gradient. As most marine animals are capable of concentrating some ions, e.g. K, against a gradient, this may have been one of the easier steps on the route to fresh water.

In the case of some of the Crustacea, Beadle and Cragg's two-stage hypothesis may well be correct, but only in the special case of animals with a low value of PA. If PA were sufficiently small, then an animal might penetrate fresh water without requiring absurdly large powers of ion absorption. If the animal is unable to replace all the ions lost in the urine the blood concentration will automatically fall, thus reducing the strain on the osmoregulatory mechanism. If the tissues can tolerate a 30% fall in the blood concentration, W will be more than halved (9). A considerable reduction in the blood concentration would have to be accompanied by changes in the osmotic constituents of the tissues, but such changes are to be classed with production of hypotonic urine as non-essential refinements. In many animals, including probably all the annelids and molluscs and possibly the smaller Crustacea, PA is so large that a drastic reduction in the blood concentration is an essential first step towards life in fresh water.

SUMMARY

1. The dynamics of osmoregulation in an ideal semi-permeable animal are discussed. The theoretical minimum osmotic work is evaluated in terms of the surface area of the animal, its permeability and the concentrations of the blood, urine and external medium.

2. It is shown that:

(*a*) The most important means whereby a marine animal entering brackish water can reduce the strain upon its osmoregulatory mechanisms is by reducing the concentration of its blood.

(*b*) In a brackish-water animal the production of urine hypotonic to the blood has only a very small effect upon the osmotic work.

(*c*) In a fresh-water animal the reduction of the urine concentration to the point at which it is isotonic with the medium can reduce the osmotic work by as much as 90%; but even a moderate reduction of the urine concentration, so that the urine is hypotonic to the blood but many times more concentrated than the medium, greatly reduces the osmotic work and is compatible with high osmoregulatory efficiency.

3. These conclusions are discussed with reference to some fresh-water animals.

I wish to thank Dr J. A. Ramsay for his criticism and advice. I am also grateful to the Department of Scientific and Industrial Research for a grant during part of the course of this work.

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THE ROLE OF PERIPHERAL NERVOUS SYSTEM IN COLOUR CHANGES OF *BUFO ARENARUM* HENSEL

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(*Received* 12 *December* 1953)

(With Plates 12 and 13)

INTRODUCTION

According to Parker (1948) the peripheral nervous system has an accessory role in the colour changes of batrachians. In the toad *Bufo arenarum* Hensel, earlier studies by Stoppani (1941, 1942*a*) lead to the same conclusion. The action of the peripheral nerves on the skin colour was however evident, and now a more detailed investigation of its mechanism and its importance in relation to the endocrine factors has been carried out.

EXPERIMENTAL

Toads (*B. arenarum* Hensel), both sexes, from 60 to 260 g. body weight, were kept at room temperature in glass containers on a white background, and used shortly after their arrival at the laboratory. Experiments were carried out from March to December.

The peripheral nervous system was stimulated by the unipolar method. The active electrode (a 5 cm. long stainless steel needle) was introduced in the spinal foramen through the space between the occipitum and the atlas, the spinal cord being destroyed. The toad was then placed on a lead sheet which acted as indifferent electrode. After several trials, diphasic current from 10 to 30 V. and 50 c./s. of 10 msec. duration, produced by an Ediswan electronic generator, was used. The efficiency of the stimuli was shown by an intense cutaneous secretion and convulsions.

Adrenalectomies were carried out by cautery or removal with the whole kidney and hypophysectomies according to Giusti & Houssay (1924). Intermedin preparations (LRW_1) were obtained as described by Landgrebe, Reid & Waring (1943). Adrenalin was from Parke Davis and Co, noradrenalin from Hoechst (I. G. Farben) and bufotenin and dehydrobufotenin (picrates) were kindly supplied by Dr Deulofeu.

Changes in the pigmentary effector system of the toad were estimated by the variation in the colour of the skin and by microscopical examination of the chromatophores. Epidermic, intracutaneous and subcutaneous melanophores were easily recognized: (1) even without staining, in sections of the skin fixed in formaldehyde (10 % w/w) or in formol-corrosive sublimate and embedded in paraffin; and (2) under oblique light, by direct microscopic examination of the deep layer of the dermis in isolated pieces of skin (mainly the subcutaneous melanophores). Sections were stained with Mayer's haemalum and eosin. The lipophores were stained by brilliant cresyl blue in sections fixed in corrosive sublimate. Guanophores were recognized by the birefringence of the guanin granules when examined under polarized light where they showed up as extremely bright points on a black background. According to Schmidt (1920) lipophores are also birefringent and consequently, might be confused with guanophores. However, in the skin of *B. arenarum*, Puente (1927) could find birefringence only in the guanophores and we have confirmed his observations in sublimate fixed skin, stained with brilliant cresyl blue (Pl. 12, figs. 1 and 2). On crossing the Nicol prisms the lipophores become dark whilst the guanophores remain brightly illuminated.

The colour of the skin has been classified into five grades, as suggested by Hogben & Gordon (1930) for the dispersion of melanocyte pigment and are: black or greenish black (5), dark green (4), green yellowish (3), light yellowish green (2), and light yellow (1). These grades correspond to the following pigment dispersion of intracutaneous melanocytes: reticular (5), with long (4), medium (3) and short (2) expansions and (1) unexpanded. Experiments were carried out in lots of no less than three animals and the figures represent the average colour (or pigment dispersion) for each lot. Individual observations did not differ more than 0·5 unit from the average.

RESULTS

(1) *Effect of stimulation of the nervous system on the colour of the toads*

The introduction of the electrode into the spinal foramen of dark toads (colour 5) produces a slight partial blanching of the skin with cutaneous secretion; if no further stimulus is applied the toads rapidly regain their dark colour. However, if the toads are then electrically stimulated for 2 min., allowed to rest for 3 min. and the stimulation is repeated in the same way for a period of 30 min., the skin becomes partially pale and secretes a viscid white liquid. The colour of the dorsum becomes 3, on the hind-legs 2·5 and on the fore-legs 1·5. Blanching is specially noticeable in the pale spots. The stimulation produces severe convulsions and if prolonged brings about heart stoppage and death, although paling does not become greater. When the stimulation ceases, the skin regains its very dark green colour (5).

At the time of maximal blanching, the skin chromatophores are found as follows.

(*a*) *Melanophores*. The subcutaneous melanophores are in a state of complete expansion (Pl. 12, fig. 3), whilst the intracutaneous melanophores (Pl. 12, fig. 5; Pl. 13, fig. 7) have reduced their dispersion to half (3) of that seen in the dark controls (dispersion 5). It must be noticed that although the subcutaneous melanophores do not seem to be affected by the stimulation, they are able to change their shape as shown by their concentration after hypophysectomy (Pl. 12, fig. 4).

(*b*) *Guanophores*. These cells are partially expanded (Pl. 13, fig. 9) in contrast with those of the dark controls which are totally contracted (Pl. 13, fig. 8) or those of the pale (hypophysectomized) toads which are completely expanded (Pl. 13, fig. 10). The changes in the guanophores are specially evident in the areas corresponding to the pale skin spots of the female and their expansion explains the more rapid paling of these areas.

(*c*) *Lipophores.* No significant changes can be detected in the lipophores which show their conventional polimorphic shape. These observations lead to the conclusion that melanophores and guanophores are the cells mainly responsible for colour changes in *B. arenarum.* It is confirmed by the fact that injection of intermedin preparations into pale hypophysectomized toads brings about darkening of the skin by expanding the melanophores and concentrating the guanophores.

In these experiments, the electric stimuli which reach the whole toad might produce: (*a*) the liberation of pituitary chromatophorotropic hormone able to lessen the effect of the nervous stimulation, or (*b*) the liberation of adrenalin (in the adrenal glands) capable of exaggerating the action of the nerves. In order to exclude these possibilities hypophysectomized toads or hypophysectomized and adrenalectomized toads, injected with sufficient intermedin to produce a submaximal darkening of the skin (colour 4), have been stimulated. The results obtained with these preparations scarcely differ from those described above. Thus, after 30 min. stimulation the colour of the skin of the dorsum is 3 and on the legs, 1·5. When stimulation ceases the colour returns to the initial state in from 15 to 60 min. This darkening of the skin is independent of the blood supply at the moment of maximal paleness (colour 3). If a piece of skin is removed or the general circulation interrupted by tying off the heart, the isolated piece of skin, or the toad with no circulation, regains the initial colour (4) in the same time as the control toads with normal circulation. The peripheral nerves are the pathways of the skin-paling stimuli since section of a lumbo-sacral plexus prevents the paling of the corresponding leg.

Results in the hypophysectomized and adrenalectomized toads injected with intermedin are somewhat similar to those obtained in the hypophysectomized toads, but the blanching is less marked which shows the importance of the adrenals for the colour changes of the toad. After 30 min. stimulation the colour on the dorsum is 3·5, on the hind-limbs 3 and on the fore-limbs, 2·5. The subcutaneous melanophores are found in dispersion 4·5, greater than would be expected by the colour of the skin (2·5). The spinal nerves are again necessary for the skin blanching, for if a lumbo-sacral trunk is sectioned the colour of the corresponding leg after stimulation is 4·0 as compared with 3·0 in the control leg. Skin regains its initial dark colour after stimulation even in isolated pieces of skin or in toads whose circulation has been arrested by cardiac ligature.

(2) *Mechanism of the action of the peripheral nerves*

In an earlier paper (Stoppani, 1941) it was observed that sympatholytic agents like F-933 inhibit the action of peripheral nerves on the colour of the skin of the toad, whilst sympathomimetics, such as cocaine exaggerate it. As adrenalin also blanches the skin it was inferred that the melanophores were controlled by adrenergic nerves. Since then, the importance of noradrenalin as a neurohumoral mediator has been demonstrated. Furthermore, the poison of *B. arenarum*, the secretion of which increases during nervous stimulation and covers the skin with a white film, has been shown to contain adrenalin (Deulofeu, 1935) and basic compounds with action partly similar to adrenalin (Chen, Jensen & Chen, 1933; Wieland, Kong & Mittasch, 1934; and Erspamer, 1952, among others). The effect of nerve stimulation on the skin colour might be due then to the following mechanisms: (1) liberation of adrenalin in a sympathetic structure, as was postulated before (Stoppani, 1941); (2) liberation of noradrenalin in the same manner; (3) action of the skin poison absorbed directly through the epidermis. Various experiments have been carried out to test these possibilities. Noradrenalin is markedly less active than adrenalin both in intensity and duration of its effect. Thus, after an injection of 1 mg./100 g. noradrenalin, the colour of the skin (initially 5) turned to 3·5 for 90 min., whilst the same amount of adrenalin blanched the skin to 1·5 for 5 hr. This agrees with the absence of noradrenalin in the skin of *B. arenarum* as has been pointed out by Cabib (1951) and therefore it is hardly likely that noradrenalin will be of physiological importance in the nervous control of the chromatophores of the toad.

Toad poison injected subcutaneously has a marked paling effect on the skin, as might be presumed from its composition, but on the other hand it has little effect when placed to act through the epidermis. This is shown by the following experiment. Toad poison (47·4 mg. wet weight) obtained by expressing parotoid glands of *B. arenarum* females, was suspended in 4·0 ml. of distilled water; half was injected into the dorsal lymph-sac of a dark toad and 1 hr. afterwards the blanching of the skin was intense. Half of the rest was rubbed with a glass rod over the leg of another dark toad and the other half was deposited on the animal's back. The leg blanched moderately at the site of the application (colour 3), but the rest of the toad suffered no colour change. Furthermore, if during electrical stimulation of dark toads, the poison film which is formed on one of the legs is removed (with a swab soaked in Ringer solution), at the end of the stimulation no colour difference is observed between the leg so treated and the control one. Experiments have also been carried out with the poison bases bufotenin and dehydrobufotenin. The injection of 2 mg. into the dorsal lymph-sac of a dark toad does not modify its colour nor does it exaggerate the paling effect of 0·5 mg. of adrenalin. These facts lead us to conclude that the cutaneous secretion plays no significant role in the colour variation of the skin of the toad.

(3) *Importance of the peripheral nerves in the colour change of* Bufo arenarum

In earlier experiments (Stoppani, 1941, 1942*a*), and in those reported in this paper, stimulation of the nervous system in dark toads did not produce an intense and rapid blanching of the skin such as follows hypophysectomy and which is attributed to disappearance of intermedin from the blood. However, hypophysectomy involves severe trauma to the toad's nervous system, whose importance in the blanching of the skin we have tried now to assess by hypophysectomizing toads: (*a*) deprived of circulation, so as to minimize variation of humoral factors (intermedin, adrenalin), and (*b*) with sectioned spinal cord, to exclude the stimulation of the caudal part of the nervous system.

(*a*) *Hypophysectomy and decapitation of toads deprived of circulation.* Circulation can be brought to a stand-still by a ligature placed (through an incision in the chest wall) round the pedicle of the heart and involving all the large vessels. This produces a partial blanching of the skin of dark toads (4·5) which colour after 1 hr. is 3·5, and after 2 hr., 2·5 (Table 1). Paling is not uniform as there are patchy light areas on the back. Toads kept lying on their backs blanch more slowly than those in their normal position, which shows the importance of the presence of blood in the skin for its colour changes. Further, in these toads the subcutaneous melanophores may be found more expanded (e.g. dispersion 4) that would correspond to the colour of the skin (e.g. colour 2·5). If immediately after ligating the large vessels the toads are hypophysectomized there is apparently no exaggerated blanching of the skin as compared with toads deprived only of their circulation, but blanching is accelerated by decapitation between the occipitum and the atlas. The latter operation is the one which produces the greatest changes in colour both in toads deprived of circulation and in normal controls. Blanching is rapid, the skin colour changing from 4·5 to 1·5 in 60 min. (Table 1). In some animals the body becomes pale quicker than the head, whilst in others the reverse obtains. There are slight changes in the blanching of the separated head, the region about the eyes being somewhat darker than the rest, but these differences are only seen in the first half hour. The presence of the pituitary in the separated head does not prevent its blanching, contrary to the claim of Juarez (1951).

Table 1. *Action of various operations on the colour of* Bufo arenarum *Hensel*

(The figures represent the average colour indexes of lots of no less than three toads. Initial skin colour, 5)

Operation	Time after the end of operation (min.)	
	60	150
(*a*) Ligature of heart pedicle (all vessels)	3·5 ± 0·5	2·5 ± 0·5
(*b*) Toracotomy	3·0 ± 0·5	3·0 ± 0·5
(*c*) Same as (*a*), plus decapitation	1·5 ± 0·5	1·0 ± 0·0
(*d*) Same as (*a*), plus craneotomy	4·0 ± 0·5	3·5 ± 0·5
(*e*) Same as (*a*), plus hypophysectomy	4·0 ± 0·5	3·5 ± 0·5
(*f*) Hypophysectomy	1·5 ± 0·5	1·0 ± 0·0

(*b*) *Hypophysectomy in toads with sectioned spinal cord.* If the pituitary is removed from toads that have had their spinal cord sectioned at the middle of the dorsum so that the colour changes in the caudal portion can be compared with the cranial as control, it is seen that the blanching is somewhat faster in the cranial portion, being most evident in the limbs 60 min. after hypophysectomy; thus whilst the colour of the fore-legs is 2, the colour of the hind-legs is 3·5 and of the dorsum is 3·0, with no significant differences in either portions of the latter. Exaggeration of the cutaneous secretion consequent to hypophysectomy can be seen only above the level of the spinal section.

DISCUSSION

It is evident that stimulation of the peripheral nerves produces a partial paling of the skin of *B. arenarum*, in agreement with previous observations (Stoppani, 1941, 1942*a*). This paling is due mainly to concentration of the pigment of the intracutaneous melanophores, and dispersion of the guanin granules in the guanophores. The action of the nervous stimuli is opposite to that of purified preparations of intermedin, which concentrates the guanin and disperses the melanin. The action of intermedin on the guanophores seems to be direct and therefore, unless separate fractions are proved in this hormone with independent actions on the melanophores and the guanophores, it should more properly be called chromatophorotropic, instead of melanotropic (or melanodispersing) as is currently done. In a recent communication Juarez (1953) has related the rapid colour changes of *B. arenarum* to variation in the amount of melanin in the skin. It is difficult to evaluate his conclusions as the appropriate evidence has not yet been produced. However, Juarez's proposition is at variance with most of the observations on the mechanism of the colour changes in batrachians (Parker, 1948) and, in the particular case of *B. arenarum*, with those of Giusti & Houssay (1924), Houssay & Ungar (1924), Puente (1927), Porto (1936) and our own. Furthermore, the important role of guanophores in the colour changes of *B. arenarum* seems to have been neglected by Juarez.

The responses of subcutaneous melanophores to nervous and humoral stimuli seem to be sluggish in comparison with those of the intracutaneous ones. In earlier papers on this subject (Stoppani, 1941, 1942*a*, *b*) the observation of subcutaneous melanophores in isolated pieces of skin was an important complement to the observation of the macroscopic changes of colour, since in *B. arenarum* there are no skin membranes like the interdigitalis in the frog, where the melanophores can be directly observed *in vivo*. Our present observations cast some doubts on the value of the subcutaneous melanophores for the evaluation of the rapid changes of the pigmentary effector system of *B. arenarum*.

The action of the nerves on the chromatophores seems to be mediated by an adrenergic-like substance since it is inhibited by sympatholytics (F-933) and exaggerated by cocaine (Stoppani, 1941). Noradrenalin and the cutaneous secretion have no apparent physiological significance. According to Juarez (1951), it is possible to blanch the skin of a dark toad by introducing underneath it a graft of skin from a pale toad, and therefore he infers the existence of a humoral agent which, under physiological circumstances, keeps the melanosomes of the hypophysectomized toads concentrated. Juarez's observations, which we have been able to confirm occasionally, may be explained by the presence of adrenalin (Deulofeu, 1935) in the cutaneous secretion of the grafted skin and do not imply a physiological role for the cutaneous secretion nor the existence in the skin of new substances that keep the melanophores contracted in the hypophysectomized toads.

Stimulation of the nervous system in *B. arenarum* does not bring about a rapid and complete paling of the skin as does hypophysectomy. This partially negative evidence does not exclude an essential role of the peripheral nerves of the toad in bringing about rapid variations of the colour of the skin. Nevertheless, previous section of the spinal cord scarcely affects the blanching of the skin after hypophysectomy, and furthermore, section of the spinal nerves does not produce significant differences in the colour variations of the denervated areas as compared with control areas (Stoppani, 1941). That leads us to assume that the paling of skin after hypophysectomy is caused mainly by the disappearance of intermedin from the blood. It is true that the interruption of the circulation blanches the skin more slowly than removal of the pituitary, but it does not prove the lack of importance of the humoral factors for the variations of the pigmentary effectors, as maintained by Juarez (1951). In this case, although the supply of intermedin ceases, its removal from the skin also comes to a standstill. This interpretation agrees with the fact that pieces of skin isolated from hypophysectomized toads, injected with intermedin and paled by electrical stimulation, recover the initial dark colour at the same time as the rest of the animal, with or without circulation. These observations also disagree with the hypothesis put forward by Juarez (1951) according to which paling of the skin by stimulation of the nerves is due to an increased uptake of intermedin *in situ*; if this were so, pieces of skin isolated from toads blanched by stimulation would not recover their initial dark colour, as actually occurs. Only decapitation in the toads without circulation produces a blanching of the skin comparable to removal of the pituitary in normal toads, but contrarily to Juarez's observations, no significant differences can be observated in the blanching of the head and the dorsum of the toad after the operation. In this case a more important role could be attributed to the stimulation of the nervous system in the blanching of the skin. However, conclusions drawn from observations in a shocked and almost bloodless preparation can hardly outweigh the well-known fact that section of the spinal or sympathetic nerves scarcely affects the physiological colour variations of batrachians (Parker, 1948) and particularly of *B. arenarum* (Stoppani, 1941, 1942*a*).

SUMMARY

1. Stimulation of the peripheral nerves of *Bufo arenarum* Hensel produces a partial paling of the skin due to concentration of pigment in the intracutaneous melanophores, and dispersion of guanin-granules in the guanophores. This effect is attributed to the liberation *in situ* of an adrenergic-like substance. Noradrenalin and the cutaneous secretion (which contains adrenalin and active bases) play no part in the blanching of the skin.

2. The nervous system plays a secondary role in the paling of the skin which follows hypophysectomy.

3. There is no evidence that stimulation of the peripheral nervous system is essential for the colour changes of *B. arenarum*.

We are much obliged to Dr V. Deulofeu for the preparation of bufotenin and dehydrobufotenin picrates, and to Dr W. Feldberg, F.R.S., for the noradrenalin.

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EXPLANATION OF PLATES

Plate 12

Fig. 1. Skin of hypophysectomized *B. arenarum*. *L*, lipophores; *M*, melanophores and *G*, guanophores. Polarized light with crossed Nicols. Brilliant cresyl blue and eosin. (× 178).

Fig. 2. Same as in fig. 1, with parallel Nicols.

Fig. 3. Skin of normal, dark, *B. arenarum*. *I*, intracutaneous and *S*, subcutaneous melanophores. Mayer's haemalum and eosin. (× 158.)

Fig. 4. Same as in fig. 3. Hypophysectomized toad.

Fig. 5. Same as in fig. 3. Normal, dark *B. arenarum* electrically stimulated.

Plate 13

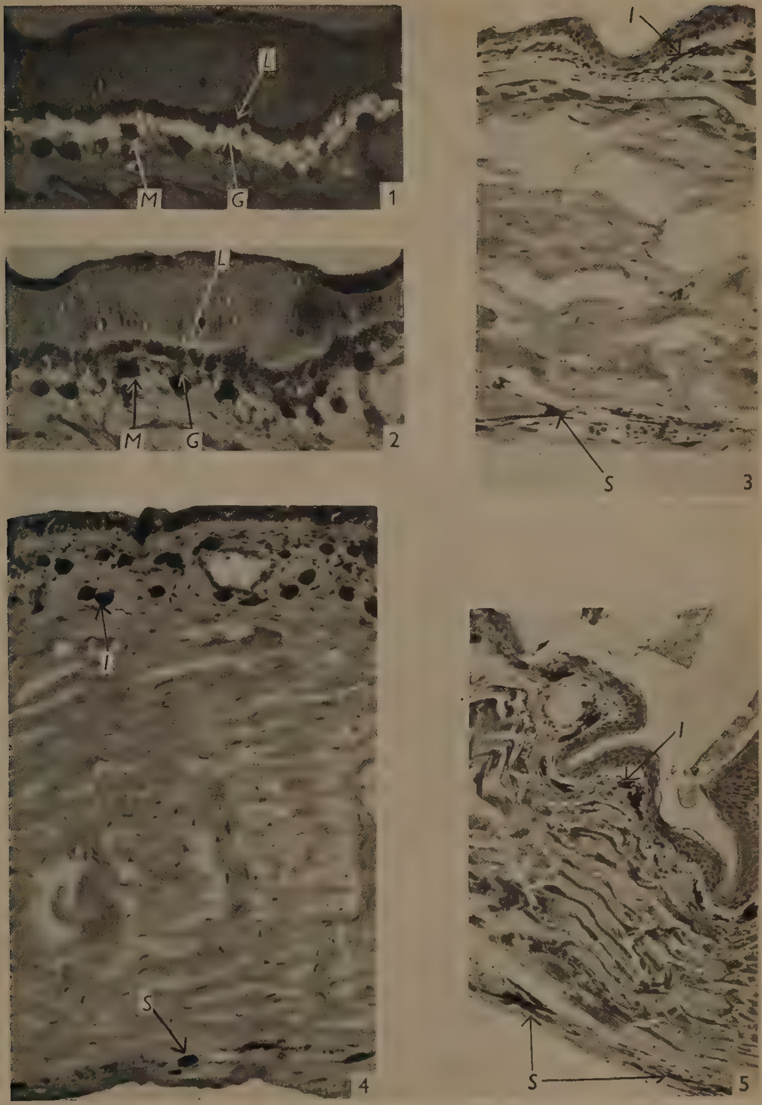
Fig. 6. Skin of normal, dark *B. arenarum*. *M*, intracutaneous melanophore, and *G*, guanophore. (× 370.)

Fig. 7. Same as in fig. 6. Electrically stimulated toad.

Fig. 8. Skin of normal dark *B. arenarum*. *G*, guanophore and *M*, melanophore. Polarized light with crossed Nicols. (× 370.)

Fig. 9. Same as in fig. 8. Electrically stimulated toad.

Fig. 10. Same as in fig. 8. Hypophysectomized toad.



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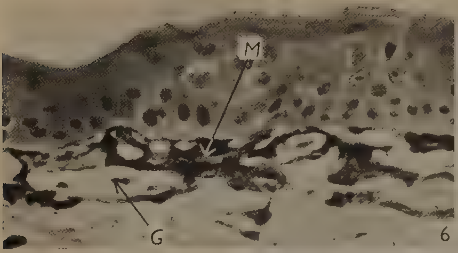

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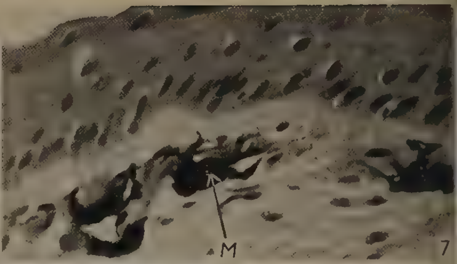

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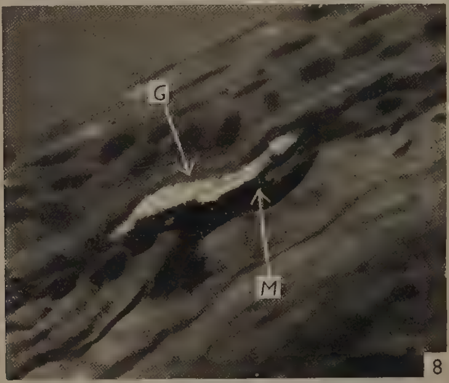

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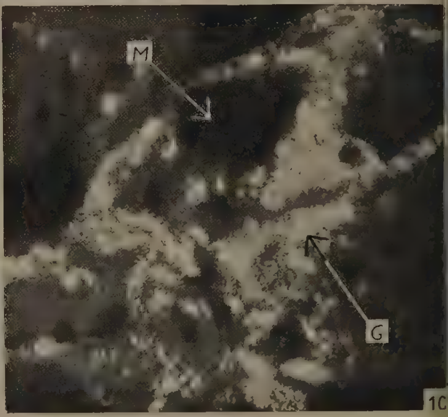

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